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Retrograde Signaling and Retrograde Axonal Transport of Leukemia Inhibitory Factor and Nerve Growth Factor By Cultured Sympathetic Neurons

(Spine Title: Retrograde Signaling and Transport)

by

Daren Raymond Ure

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, "Retrograde Signaling and Retrograde Axonal Transport of Leukemia Inhibitory Factor and Nerve Growth Factor by Cultured Sympathetic Neurons" submitted by Daren Raymond Ure in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Robert B. Campenot (Supervisor)

Dr. Larry Guilbert

Dr. Dennis Vance

Dr. Tessa Gordon

Dr. Mark Bothwell (External Examiner)

DEDICATION

I dedicate this thesis to my parents who taught me the value of hard work and commitment.

ABSTRACT

This thesis firstly examines the retrograde signaling and retrograde transport of leukemia inhibitory factor (LIF) and secondly the retrograde transport of nerve growth factor (NGF) in rat sympathetic neurons grown in compartmented cultures. LIF, previously known to induce a cholinergic phenotype in cultured sympathetic neurons, is demonstrated here to be capable of inducing this phenotype through retrograde signaling, as assayed by changes in neurotransmitter enzyme activity and quantity in cell bodies/proximal axons. The retrograde effects of LIF were observed to be concentration-dependent and time-dependent. Radioiodinated LIF was retrogradely transported following binding to specific receptors, but to lower levels than NGF transport. However, the time course of LIF transport was similar to that previously reported for NGF, and also in similarity to NGF a significant portion of transported LIF appeared to be intact in cell bodies/proximal axons. These results are consistent with the possibility that LIF transport could participate in retrograde signaling. Examining NGF transport, I demonstrate that radioiodinated NGF was transported at a higher velocity than previously reported, and virtually no NGF or its degradation products were released from axons during retrograde transport. Contrasting these efficient transport parameters, the rate of NGF transport was relatively low, which appeared to reflect a low internalization rate because most axonal NGF was surface-bound at steady-state. A consequence of the low transport rate was that most cell-associated NGF was not in cell bodies under steady-state transport conditions but rather was associated with distal axons. Slowly-dissociating receptors, whose binding correlated strongly with NGF retrograde transport, were not scarce on distal axons, suggesting that the transport rate was limited primarily by factors other than the abundance of transport-competent receptors. The NGF receptor of most importance to NGF transport was trkA. Discrepant effects of anti-p75 treatments prevented

resolution of the role of p75 in NGF transport but raised the possibility that p75 might be capable of regulating retrograde transport of trkA-NGF complexes. Insights gained from these studies will be valuable for future studies analyzing whether ligand transport or, alternatively, other mechanisms are responsible for retrograde signaling.

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LIST OF ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CDF	cholinergic differentiation factor
ChAT	choline acetyltransferase
CNTF	ciliary neurotrophic factor
DFCM	dermal fibroblast conditioned medium
HCCM	heart cell conditioned medium
K_D	steady state dissociation constant
LIF	leukemia inhibitory factor
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4	neurotrophin-4
PBS	phosphate-buffered saline
PC12	pheochromocytoma-12
SCG	superior cervical ganglion
TH	tyrosine hydroxylase

Chapter 1

Background and Introduction

BACKGROUND

Retrograde Axonal Signaling

The molecular environment into which neurons extend their axons can be significantly different from the environment around cell bodies. The molecular information about the neuronal targets is communicated to cell bodies by retrograde axonal signaling. Retrograde signals in turn influence events localized in cell bodies such as mRNA and protein synthesis. Perhaps the best example of the importance of retrograde signaling is the regulation of naturally-occurring cell death which is a characteristic of many neuronal populations during development (Purves and Lichtman, 1985). Here it is believed that the magnitude of the retrograde response to peripheral neurotrophic cues determines the extent of neuronal survival during a critical developmental period. The magnitude of the retrograde response is itself thought to reflect competition by axons for limited supplies of target-derived trophic factor. In addition to the regulation of survival, retrograde signals can also influence neuronal morphology, phenotype, and metabolic activity, not only during development but also in adult organisms (Schwab and Thoenen, 1982; Black, 1986; Oppenheim, 1989; Kuno, 1990; Svendsen et al., 1991; Landmesser, 1992; Hendry, 1993; Vogel, 1993; Marty and Peschanski, 1995; Jeanpretre et al., 1996).

Ligand-Receptor Transport Hypothesis of Retrograde Signaling

The mechanism by which target-derived signals reach cell bodies is not well understood, and indeed many types of mechanisms might exist. The type of retrograde signal which has been most extensively studied is that generated by secreted molecules which bind to receptors on the axonal surface. For this type of signal, it has been most commonly hypothesized that ligand-receptor complexes comprise the retrograde signal (for reviews, see Hendry, 1993; Campenot, 1994). According to this hypothesis, ligand-receptor complexes generated on axons are internalized and become constituents of vesicles or other types of organelles. The organelles

become associated with the microtubule-based transport mechanism in axons and are then carried to cell bodies. In cell bodies the catalytic activity of the receptor is believed to be maintained, most likely by ligand binding, thereby generating signaling cascades directly in cell bodies. The retrograde transport of NGF by sympathetic neurons has been the most influential model in formulating this hypothesis and is discussed below. NGF transport has widely been interpreted as participating in, and in turn reflecting the retrograde signaling mechanism. In addition to NGF, many other molecules which promote survival or phenotypic responses in specific neurons in culture have been demonstrated to be retrogradely transported in vivo (Hendry and Hill, 1980; Curtis et al., 1992, 1993, 1995; DiStefano et al., 1992; Tomac et al., 1995; Von Bartheld et al., 1996). No studies have sufficiently challenged the theory that accumulation of target-derived signaling molecules in cell bodies is what is largely responsible for retrograde signaling.

The Cholinergic Switch

A well characterized example of retrograde signaling is the change in neurotransmitter phenotype of sympathetic neurons which innervate sweat glands of the rat (Landis, 1990; Schotzinger et al., 1994). These neurons are noradrenergic (i.e. secrete noradrenaline) in newborn rats but a short time later this phenotype almost completely disappears and the neurons begin to secrete acetylcholine. Hence, the change in phenotype is called the cholinergic switch. Changes in neuropeptide phenotype also occur. The sympathetic innervation of the footpads, where most of the sweat glands are located, is noradrenergic until approximately postnatal day 10. The phenotypic switch then progresses for about the next two weeks, after which the mature cholinergic phenotype predominates.

The cholinergic switch is a target-specified event, as demonstrated best by target exchange experiments. When Schotzinger and Landis (1988) transplanted sweat glands into the thorax of neonatal rats, the glands became innervated by noradrenergic sympathetic axons which normally innervated the hairy skin of the thorax and which normally remained noradrenergic throughout life. At about 3 weeks post-transplantation, it was found, however, that the catecholamine fluorescence of the axons innervating the sweat gland, but not of the axons innvervating the surrounding hairy skin, began to gradually decrease. Concurrently, the choline acetyltransferase activity (indicative of cholinergic innvervation) dramatically increased,

suggesting that the sweat glands induced the change in neuronal phenotype. In reciprocal experiments Schotzinger and Landis (1990) found that when parotid glands (which normally have only noradrenergic innervation) were substituted for sweat glands in the footpads of neonatal rats, the sympathetic innervation to the footpad did not undergo the cholinergic switch which would normally occur, but rather remained adrenergic, which again reflected target-mediated control of phenotype.

In vivo observations of the cholinergic switch were, in fact, preceeded by similar observations of cholinergic switching in cultured sympathetic neurons in response to various applied factors. Patterson and Chun (1977) demonstrated first that the switch occurs in response to conditioned medium from a variety of cultured cells. The switching factor from cultured heart cells, called cholinergic differentiation factor or CDF, was characterized over the next several years. Eventually its amino acid sequence was determined (Yamamori et al., 1989), which revealed that CDF is the same as leukemia inhibitory factor (LIF).

In addition to its effects on neurotransmitter phenotype, CDF/LIF also is capable of supporting the survival of a subset of sympathetic neurons cultured in the presence of nonneuronal cells. CDF/LIF has been found to independently support 70% of the neurons normally supported by NGF, but the responsiveness to CDF/LIF does not begin to develop until approximately postnatal day 3 (Kotzbauer et al., 1994). Interestingly, LIF has also been reported to induce apoptotic death in the same neurons (Kessler et al., 1993). Kotzbauer et al. (1994) found that even in the presence of NGF approximately 30% of the neurons died in response to CDF/LIF. CDF/LIF has also been shown to provide trophic support for sensory, motor, and nodose neurons (Martinou et al., 1992; Murphy et al., 1991, 1993; Thaler et al., 1994) as well as to exert a wide variety of effects in many organs outside the nervous system (Hilton and Gough, 1991; Kurzrock et. al., 1991).

Other molecules also induce the cholinergic switch, including ciliary neurotrophic factor (CNTF; Saadat et. al., 1989) and 2 spinal cord membrane-associated factors (MANS; Wong and Kessler, 1987; Adler et. al., 1989). These molecules as well as others such as interleukin-6 and oncostatin M have related effects because they bind to one or more of the same receptors belonging to the hematopoietin receptor family (Yamamori and Sarai, 1994; Shields et al., 1995). The commonly-shared, signal-transducing receptor of this family is the tyrosine kinase, gp130 (Gearing et al., 1992; Ip et al., 1992a; Taga et al., 1992). CDF/LIF appears to be able to bind

directly to either gp130 or to a second related subunit termed LIF receptor-β (Gearing et al., 1991; Modrell et al., 1994), but the heteromeric complex of LIF, LIFR-β, and gp130 appears to most effectively transduce the CDF/LIF signal (Baumann et al., 1994; Gearing et al., 1994).

It has now been reported that CDF/LIF is not the cholinergic factor in rat footpads. Rather the footpad factor appears to represent at least 2 factors, the primary one being CNTF and the other being a related but unidentified factor (Rao and Landis, 1990; Rao et al., 1992). However, CDF/LIF is reported to be responsible for the changes in neuropeptide phenotype in sympathetic neurons resulting from axotomy (Rao et al., 1993). This role for LIF is consistent with the observation that LIF mRNA levels increase dramatically at the site of sciatic nerve transection (Banner and Patterson, 1994; Curtis et al., 1994).

NGF Retrograde Signaling and Retrograde Transport

Nerve growth factor (NGF) is the best characterized neurotrophic factor. Its best known action is its ability to maintain the survival of sympathetic neurons, some dorsal root ganglion sensory neurons, and some neurons of the central nervous system. Reviews on NGF, its receptors, and its actions are widely available (Levi-Montalcini, 1976; Greene and Shooter, 1980; Thoenen and Barde, 1980; Thoenen et. al., 1987; Barker and Murphy, 1992; Meakin and Shooter, 1992; Bradshaw et al., 1993; Chao and Hempstead, 1995; Rush et. al., 1995).

Discovery of the neurotrophic activity of NGF dates back to 1948 when it was discovered that the mouse sarcoma tumor 180, when grafted onto the body wall of 3-day-old chick embryos, resulted in an increase in the size of the dorsal root ganglia that innervated the tumor (Bueker, 1948). A similar observation was made by Levi-Montalcini and Hamburger (1951), who repeated the experiments, but additionally Levi-Montalcini and Hamburger observed an increase in the size of sympathetic ganglia. These effects were found to result from a diffusible agent released by the tumor, since implantation of the tumor onto the chorioallantoic membrane of developing chicks also resulted in an increase in ganglion size and neurite outgrowth in the embryos (Levi-Montalcini, 1952). Neurite outgrowth from explanted sensory or sympathetic ganglion maintained in a hanging plasma clot was developed as the in vitro assay for the nerve growth activity (Levi-Montalcini et al., 1954). The growth activity was given the name, nerve

growth factor, in 1954 when Cohen et al. (1954) isolated the activity within a nucleoprotein fraction of the sarcoma tumors 180 and 37. To further purify the crude extract, Cohen and Levi-Montalcini (1956) performed experiments in which snake venom was added to the nucleoprotein fraction, reasoning that the phosphodiesterase from the venom would cleave the nucleic acids. Unexpectedly, the snake venom increased neurite outgrowth, which was later shown to be due to the presence of NGF in snake venom (Hogue-Angeletti et al., 1976). The presence of NGF was then sought in mammalian salivary glands, which are homologous to snake venom glands, and high concentrations of NGF indeed were found in male mouse submandibular glands (Cohen, 1960). To present day no other tissues that have been examined have been found to produce more NGF than male mouse submandibular gland, but the role of so much NGF from this source still remains an enigma. The amino acid sequence of the salivary gland NGF was finally determined in 1971 (Hogue-Angeletti and Bradshaw, 1971). A more detailed, historical account of the discovery and characterization of NGF can be found elsewhere (Levi-Montalcini, 1975). For their instrumental work on NGF, Rita Levi-Montalcini and Stanley Cohen received the Nobel Prize in Medicine in 1986.

NGF is expressed in the targets of NGF-responsive neurons, supportive of a role in retrograde signaling, but it is additionally expressed outside of the nervous system, suggesting that it has functions other than retrograde signaling (Korsching and Thoenen, 1983b, 1988; Shelton and Reichardt, 1984, 1986; Whitemore et al. 1986; Nagata et al., 1987; Ayer-LeLievre et al., 1988; Wheeler and Bothwell, 1992). In vivo demonstrations of retrograde signaling by NGF include: a) increases in neuron size, tyrosine hydroxylase activity, and mRNA expression in the rat superior cervical ganglion (SCG) following injection of NGF into targets of the SCG (Hendry, 1977; Paravicini et al., 1975; Stoeckel and Thoenen, 1975; Miller et al., 1994); b) increases in substance P and protein content of sensory ganglia following injection into a sensory neuron target (Goedert et al., 1981). In compartmented cultures of sympathetic neurons retrograde signaling is demonstrated by the observation that the neurons will survive if NGF is absent from medium bathing cell bodies but is present in medium bathing distal axons (Campenot, 1977). Some studies have also interpreted a role for NGF in retrograde signaling from observations that the retrograde effects of axotomy or treatment with agents which disrupt microtubule-based axonal transport in sympathetic neurons can be reversed by systemic administration of NGF (Hendry, 1975; Purves, 1976; Chen et al., 1977; Johnson, 1978).

Findings on NGF retrograde transport have significantly contributed to the development of the ligand-receptor hypothesis of retrograde signaling. The discovery that NGF is retrogradely transported was first made over 20 years ago, using as a model the sympathetic innervation of the adult rat iris. Radioiodinated NGF injected into the anterior chamber of the eye was found to be taken up by axons and delivered by axonal transport to the corresponding cell bodies of the superior cervical ganglion (Hendry et al., 1974a,b; Stöckel et al., 1974; Stöckel and Thoenen, 1975; Johnson et al., 1978; Dumas et al., 1979). Retrograde transport of endogenous NGF was confirmed later (Korsching and Thoenen, 1983a; Palmatier et al., 1984; Nagata et al., 1987), as was NGF retrograde transport by the other neurons (Stoeckel et al., 1975; Richardson and Riopelle, 1984; Johnson et al., 1987; Wayne and Heaton, 1988; Yan et al., 1988). Transported NGF in axons is found within multivesicular bodies, smooth vesicles, lysosomes and other organelles, while in cell bodies it is contained largely within lysosomes (Schwab, 1977; Claude et al., 1982). Original studies showed NGF to be much more concentrated in sympathetic ganglia than in their target tissues, which was reported to reflect its accumulation resulting from retrograde transport rather than local synthesis of NGF in the SCG (Korsching and Thoenen, 1983b, 1985, 1988; Nagata et al., 1987). However, a recent study suggests that the concentration of NGF in some target tissues is higher than previously reported, so differences between target and ganglionic levels of NGF might not be as disparate as previously thought (Zettler et al., 1996). NGF in cell bodies is reported to be mostly intact both structurally and antigenically (Hendry et al., 1974a; Stöckel et al., 1974, 1976; Johnson et al., 1978; Dumas et al., 1979).

Binding of NGF to its receptor is required for NGF to exert biological effects, as suggested by the findings that NGF microinjected directly into the cytoplasm of PC12 cells does not increase ChAT activity but that NGF will increase ChAT activity when it is supplied to the bathing medium (Heumann et al., 1984). The two known receptors for NGF are the trkA tyrsosine kinase (Barbacid, 1994) and the p75 neurotrophin receptor (Chao, 1994). trkA, the second of the two receptors to have been cloned (Kaplan et al., 1991; Klein et al., 1991), belongs to a family of tyrosine kinase receptors. Other members of this receptor family include trkB and trkC, each showing specificity for other neurotrophins. trkA binds NGF with high specificity, although it will also bind neurotrophin-3 (NT-3; Dechant et al., 1993) and neurotrophin-4 (NT-4; Berkemeier et al., 1991; Ip et al., 1992b) to a limited extent. NGF is reported to bind to trkA mostly with both low affinity ($K_D \approx 10^{-9}$ M), although some studies have also reported a small

fraction which binds with high affinity ($K_D \approx 10^{-11}$ M; Kaplan et al., 1991; Klein et al., 1991; Jing et al., 1992; Mahadeo et al., 1994). trkA confers NGF-responsiveness to cells and does not require the second NGF receptor, p75, to elicit the survival and growth-promoting effects of NGF (Loeb et al., 1991; Hartman and Hertel, 1994; Smeyne et al., 1994; Verdi et al., 1994a; Lucidi-Phillipi et al., 1996). trkA tyrosine kinase activity and receptor interphosphorylation presumably results from trkA dimerization induced by binding of the NGF dimer (Jing et al., 1992).

The first NGF receptor to be cloned was p75 (Johnson et al., 1986; Radeke et al., 1987) but it still is the less understood of the two NGF receptors. p75 is a glycoprotein belonging to a family of receptors which also includes receptors for tumor necrosis factor, fas antigen, and CD40 (Chao, 1994). p75 binds NGF with low affinity ($K_d = 10^{-9}$ M), but also binds other neurotrophins with low affinity, including brain-derived neurotrophic factor (BDNF; Rodriguez-Tébar et al., 1990), NT-3 (Rodriguez-Tébar et al., 1992), NT-4 (Timm et al., 1994), and likely neurotrophin-6 (NT-6; Gotz et al., 1994). Most studies investigating p75 action report that p75 promotes NGF high affinity binding (Hempstead et al., 1990, 1991; Battleman et al., 1993l; Mahadeo et al., 1994) and augments trkA activity (Barker and Shooter, 1994; Verdi et al., 1994b). However, p75 might also function more independently by participating in apoptotic signaling (Barret and Bartlett, 1994; Rabizadeh and Bredesen, 1994) or other distinct signaling cascades (Carter et al., 1996).

trkA has been shown to be retrogradely transported (Loy et al., 1994), and its phosphorylation state in axons reflects the availability of NGF for binding to axon terminals (Ehlers et al., 1995). The latter implies most likely that NGF is bound to trk en route to cell bodies, although perhaps trkA phosphorylation generated more distally in axons is sustained during transport by a mechanism other than NGF binding. The involvement of p75 in NGF transport has been less clear. Studies have shown p75 to be retrogradely transported in sympathetic and other neurons (Taniuchi and Johnson, 1985; Yan et al., 1988; Johnson et al., 1987). On the other hand, Curtis et al. (1995) reported that p75 binding is of little importance for NGF retrograde transport. In PC12 pheochromocytoma cells, which express p75 and trkA and which have been used extensively for studying NGF's actions, NGF internalization is reported not to involve p75 binding (Kahle et al., 1994). In nonneuronal cells not expressing trkA, p75 has been shown to internalize NGF, albeit at lower levels than when trkA is expressed alone (Le Bivic et al., 1991; Kahle and Hertel, 1992; Mahadeo et al., 1994).

INTRODUCTION

Experimental Model: Compartmented Cultures of Rat Sympathetic Neurons

The experimental model used for all experiments in this thesis was compartmented cultures of rat sympathetic neurons. The most common design was a 3-compartment design, shown diagramatically in Figure 1.1. Briefly, superior cervical ganglia were removed from newborn rats, enzymatically and mechanically dissociated, and the cell bodies plated into a central compartment of a 3-compartment, Teflon divider seated onto a collagen substrate. Axons extended along tracks made in the collagen substrate, under the Teflon divider, and into left and right side compartments where extensive axon networks developed. Thus, the 2 basic types of compartments contained either: a) cell bodies and proximal axons, or; b) distal axons alone. Because diffusion under the Teflon divider occurred at an extremely low rate, the fluid environments of compartments were effectively separated. Therefore, LIF or NGF, either in unlabeled or radioiodinated form, could be applied to distal axons, and their retrograde effects or transport to cell bodies/proximal axons analyzed.

Investigating retrograde transport of ligands in compartmented cultures has provided several advantages over in vivo models which have traditionally been used to investigate ligand transport. In compartmented cultures distal axons can be bathed continuously with the molecule of interest, permitting not only retrograde transport but also association of the molecule with axons at steady-state to be measured. A precise estimate of all the ligand that is retrogradely transported has been possible because, in addition to ligand which accumulates in cell bodies, the degraded ligand released from cell bodies can also be collected, unlike in animal models where degradation products are lost to the circulation. Finally, the culture design can be varied (e.g. addition of extra compartments) in order to experimentally address specific questions.

Hypotheses

Chapter 2

At the time that the study in Chapter #2 were undertaken, the molecule(s) in rat sweat glands which elicits the cholinergic switch by retrograde signaling had not been identified, but CDF/LIF was a candidate for mediating this event. However, it was not known whether CDF/LIF could signal the change in neurotransmitter phenotype by retrograde signaling. Moreover, retrograde signaling by any exogenous ligand had been directly demonstrated only in the case of NGF. Therefore, the following hypothesis was formulated:

Hypothesis #1: That CDF/LIF could evoke the cholinergic switch by retrograde signaling.

Chapter 3

Having demonstrated retrograde signaling by CDF/LIF, I next investigated whether LIF could be retrogradely transported. This was of interest because of the long-standing theory, based on NGF transport studies, that vesicle-associated, ligand-receptor complexes act as retrograde messengers. Moreover, if LIF was transported, I was interested in whether its transport was comparable to that of NGF, or affected by NGF, since NGF has been the prototype of a retrogradely transported signaling molecule.

Hypothesis #2: That ^{125}I-LIF could be retrogradely transported following binding to specific receptors and would accumulate intact in cell bodies.

Chapter 4

If the retrograde accumulation of NGF in cell bodies is important for retrograde signaling, then a reasonable prediction is that receptor-bound NGF on axons would be delivered efficiently to cell bodies and degraded slowly, resulting in a large accumulation of NGF in cell bodies. Early studies on NGF distribution and transport in vivo have been consistent with this prediction (Korsching and Thoenen, 1983b, 1985; Nagata et al., 1987), although more recent observations shed some doubt on this view (Liu et al., 1996). Moreover, a detailed analysis of

transport rates, degradation rates, and steady-state distribution of NGF in neurons has not been performed. Thus, using compartmented cultures we addressed the following hypotheses:

Hypothesis #3: That NGF bound to receptors on axons would be delivered at a high rate to cell bodies.

Hypothesis #4: That NGF would be rapidly internalized and transported at a high velocity on the retrograde transport mechanism.

Hypothesis #5: That NGF would not be degraded in axons or released intact from axons while en route to cell bodies.

Hypothesis #6: That during incubation of NGF with distal axons, most neuron-associated NGF would represent a retrograde accumulation in cell bodies/proximal axons rather than association with distal axons.

Hypothesis #7: That NGF in cell bodies would be degraded with a relatively slow half-life.

Chapter 5

Determining to what extent trkA and p75 receptors were involved in NGF retrograde transport was of interest because the 2 receptors are very different in structure and activity, which might affect the nature of retrograde signaling. In support of a role for p75 in retrograde transport, p75 has been reported to be involved in NGF high affinity binding (Hempstead et al., 1990, 1991; Battleman et al., 1993; Mahadeo et al., 1994; Weskamp and Reichardt, 1991), and in turn high affinity binding has been reported to participate in retrograde transport (Dumas et al., 1979). Also, p75 is retrogradely transported, as are large quantities of the MC192 antibody to p75 (Taniuchi and Johnson, 1985; Johnson et al., 1987; Yan et al., 1988). In contrast to this support, Curtis et al. (1995) did not find a significant role for p75 in NGF transport by sympathetic neurons in vivo.

Hypothesis #8: That not only trkA but also p75 would be involved in NGF retrograde transport.

REFERENCES

Adler JE, Schleifer LS, Black IB (1989) Partial purification and characterization of a membrane-derive factor regulating neurontransmitter phenotypic expression. Proc Natl Acad Sci USA 86:1080-1083.

Ayer-LeLievre C, Olson L, Ebendal T, Hallböök F, Persson H (1988) Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. Proc Natl Acad Sci USA 85:2628-2632.

Banner LR, Patterson PH (1994) Major changes in the expression of the mRNAs for cholinergic differentiation factor-leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. Proc Natl Acad Sci USA 91:7109-13.

Barbacid M (1994) The trk family of neurotrophin receptors. J Neurobiol 25:1386-1403.

Barker PA, Murphy RA (1992) The nerve growth factor receptor: a multicomponent system that mediates the actions of the neurotrophin family of proteins. Mol Cell Biochem 110:1-15.

Barker PA, Shooter EM (1994). Disruption of NGF binding to the low affinity neurotrophin receptor $p75^{LNTR}$ reduces NGF binding to trkA on PC12 cells. Neuron 13:203-215.

Barret GL, Bartlett PF (1994) The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. Proc Natl Acad Sci USA 91:6501-6505.

Battleman DS, Geller AI, Chao MV (1993) HSV-1 vector-mediated gene transfer of the human nerve growth factor receptor $p75^{hNGFR}$ defines high-affinity NGF binding. J Neurosci 13:941-951.

Baumann H, Symes AJ, Comeau MR, Morella KK, Wang Y, Friend D, Ziegler SF, Fink JS, Gearing DP (1994) Multiple regions within the cytoplasmic domains of the leukemia inhibitory factor receptor and gp130 cooperate in signal transduction in hepatic and neuronal cells. Mol Cell. Biol 14:138-146.

Berkemeier LR, Winslow JW, Kaplan DR, Kikolics K, Goeddel DV, Rosenthal A (1991) Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. Neuron 7:857-866.

Black IB (1986) Trophic molecules and evolution of the nervous system. Proc Natl Acad Sci USA 83:8249-8252.

Bradshaw RA, Blundell TL, Lapatto R, McDonald NQ, Murray-Rust J (1993) Nerve growth factor revisited. TIBS 18:48-52.

Bueker ED (1948) Implantation of tumors in the hindlimb field of the embryonic chick and developmental response of the lumbosacral nervous system. Anat Rec 102:369-390.

Campenot RB (1977) Local control of neurite development by nerve growth factor. Proc Natl Acad Sci USA 74:4516-4519.

Campenot RB (1994) NGF and the local control of nerve terminal growth. J Neurobiol 25:599-611.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhäuser N, Böhm-Matthaei R, Baeuerle PA, Barde Y-A (1996) Selective activation of NF-κB by nerve growth factor through the neurotrophin receptor p75. Science 272:542-545.

Chao MV (1994) The p75 neurotrophin receptor. J Neurobiol 25:1373-1385.

Claude P, Hawrot E, Parada I (1982) Ultrastructural studies on the intracellular fate of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J Cell Biochem 20:1-13.

Chao MV, Hempstead BL (1995) p75 and Trk: a two-receptor system. TINS 18:321-326

Chen MGM, Chen JS, Calissano P, Levi-Montalcini R (1977) Nerve growth factor prevents vinblastine destructive effects on sympathetic ganglia in newborn mice. Proc Natl Acad Sci USA 74:5559-5563.

Cohen S (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. Proc Natl Acad Sci USA 46:302-311.

Cohen S, Levi-Montalcini R (1956) A nerve growth stimulating factor isolated from snake venom. Proc Natl Acad Sci USA 42:571-574.

Cohen S, Levi-Montalcini R, Hamburger V (1954) A nerve growth-stimulating factor isolated from sarcomas 37 and 180. Proc Natl Acad Sci USA 40:1014-1018.

Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, DiStefano PS (1993) Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature 365:253-255.

Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, Zhu Y, Lindsay RM, DiStefano PS (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: Correlation with increased LIF expression in distal nerve. Neuron 12:191-204.

Curtis R, Adryan KM, Stark JL, Park J, Compton DL, Weskamp G, Huber LJ, Chao MV, Jaenisch R, Lee K-F, Lindsay RM, DiStefano PS (1995) Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14:1201-1211.

Dechant G, Rodriguez T, Kolbeck R, Barde Y-A (1993) Specific high-affinity receptors for neurotrophin-3 on sympathetic neurons. J Neurosci 13:2610-2613.

DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ (1992) The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8:983-993.

Dumas M, Schwab ME, Thoenen H (1979) Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. J Neurobiol 10:179-197.

Ehlers MD, Kaplan DR, Price DL, Koliatsos,VE (1995) NGF-stimulated retrograde transport of trkA in the mammalian nervous system. J Cell Biol 130:149-156.

Gearing DP, Thut CJ, VandenBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman D, Beckmann MP (1991) Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J 10:2839-2848.

Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasher KK, King JA, Gilllis S, Mosley B, Ziegler SF, Cosman D (1992) The IL-6 signal transducer, gp130: An oncostatin M receptor and affinity converter for the LIF receptor. Science 255:1434-1437.

Gearing DP, Ziegler SF, Comeau MR, Friend D, Thoma β, Cosman D, Park L, Mosley β (1994) Proliferative responses and binding properties of hematopoietic cells transfected with low-affinity receptors for leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor. Proc Natl Acad Sci USA 91:1119-23.

Goedert M, Stoeckel K, Otten U (1981) Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons. Proc Natl Acad Sci USA 78:5895-5898.

Gotz R, Koster R, Winkler C, Raulf F, Lottspeich F, Schartl M, Thoenen H (1994) Neurotrophin-6 is a new member of the nerve growth factor family. Nature 372:266-269.

Greene LA, Shooter EM (1980) The nerve growth factor: Biochemistry, synthesis, and mechanism of action. Ann Rev Neurosci 3:353-402.

Hartman DS, Hertel C (1994) Nerve growth factor-induced differentiation in neuroblastoma cells expressing trkA but lacking $p75^{NGFR}$. J Neurochem 63:1261-1270.

Hempstead BL, Patil N, Thiel B, Chao MV (1990) Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding. J Biol Chem 265:9595-9598.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coespression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature 350:678-683.

Hendry IA (1975) The response of adrenergic neurones to axotomy and nerve growth factor. Br Res 94:87-97.

Hendry IA (1977) The effect of the retrograde axonal transport of NGF on the morphology of adrenergic neurones. Br Res 134:213-223.

Hendry IA (1993) Retrograde factors in peripheral nerves. Pharmac Ther 56:265-285.

Hendry IA, Hill CE (1980) Retrograde axonal transport of target-derived macromolecules. Nature 287:647-649.

Hendry IA, Stockel K, Thoenen H, Iversen LL (1974a) The retrograde axonal transport of nerve growth factor. Br Res 68:103-121.

Hendry IA, Stach R, Herrup K (1974b) Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. Br Res 82:117-128.

Heumann R, Schwab M, Merkl R, Thoenen H (1984) Nerve growth factor-mediated induction of choline acetyltransferase in PC12 cells: Evaluation of the site of action of nerve growth factor and the involvement of lysosomal degradation products of nerve growth factor. J. Neurosci. 4:3039-3050.

Hilton DJ, Gough NM (1991) Leukemia inhibitory factor: A biological perspective. J Cell Biochem 46:21-26.

Hogue-Angeletti R, Bradshaw RA (1971) Nerve growth factor from mouse submaxillary gland: amino acids sequence. Proc Natl Acad Sci USA 68:2417-2420.

Hogue-Angeletti R, Frazier WA, Jacobs JW, Niall RD, Bradshaw RA (1976) Purification, characterization and partial amino acid sequence of nerve growth factor from cobra venom. Biochemistry 15:35-39.

Ip NY, Nye SH, Boulton TG, Davis S, Taga T, Li Y, Birren SJ, Yasukawa K, Kishimoto T, Anderson DJ, Stahl N, Yancopoulous GD (1992a) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell 69:1121-1132.

Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa III R, Squinto SP, Persson H, Yancopoulos GD (1992b) Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 89:3060-3064.

Jing S, Tapley P, Barbacid M (1992) Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron 9:1067-1079.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, and Chao MV (1986) Expression and structure of the human NGF receptor. Cell 47:545-554.

Johnson, EM Jr (1978) Destruction of the sympathetic nervous system in neonatal rats and hamsters by vinblastine: Prevention by concomitant administration of nerve growth factor. Br Res 141:105-118.

Johnson EM Jr, Andres RY, Bradshaw RA (1978) Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I]NGF. Br Res 150:319-331.

Johnson EM Jr, Taniuchi M, Clark HB, Springer JE, Koh S, Tayrien MW, and Loy R (1987) Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. J Neurosci 7:923-929.

Kahle P, Hertel C (1992) Nerve growth factor (NGF) receptor on rat glial cell lines: Evidence for NGF internalization via $p75^{NGFR}$. J Biol Chem 267:13917-13923.

Kahle P, Barker PA, Shooter EM, Hertel C (1994) p75 nerve growth factor receptor modulates $p140^{trkA}$ kinase activity, but not ligand internalization, in PC12 cells. J Neurosci Res 38:599-606.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991) The trk proto-oncogene product: A signal transducing receptor for nerve growth factor. Science 252:554-558.

Kessler JA, Ludlam WH, Frdidin MM, Hall DH, Michaelson MD, Spray DC, Dougherty M, Batter DK (1993) Cytokine-induced programmed death of cultured sympathetic neurons. Neuron 11:1123-1132.

Klein R, Jing, Nanduri V, O-Rourke E, Barbacid M (1991) The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Korsching S, Thoenen H (1983a) Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. Neurosci Letts 39:1-4.

Korsching S, Thoenen H (1983b) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Corrleation with density of sympathetic innervation. Proc Natl Acad Sci USA 80:3513-3516.

Korsching S, Thoenen H (1985) Treatment with 6-hydroxydopamine and colchicine decreases nerve growth factor levels in sympathetic ganglia and increases them in the corresponding target tissues. J Neurosci 5:1058-1061.

Korsching S, Thoenen H (1988) Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev Biol 126:40-46.

Kotzbauer PT, Lampe PA, Estus S, Milbrandt J, Johnson EM Jr (1994) Postnatal development of survival responsiveness in rat sympathetic neurons to leukemia inhibitory factor and ciliary neurotrophic factor. Neuron 12: 763-773.

Kuno M (1990) Target dependence of motoneuronal survival: the current status. Neurosci Res 9:155-172.

Kurzrock R, Estrov Z, Wetzler M, Gutterman JU, Talpaz M (1991) LIF: Not just a leukemia inhibitory factor. Endocr Rev 12:208-216.

Landis SC (1990) Target regulation of neurotransmitter phenotype. TINS 13:344-350.

Landmesser L (1992) The relationship of intramuscular nerve branching and synaptogenesis to motoneuron survival. J Neurobiol 23:1131-1139.

Le Bivic A, Sambuy Y, Patzak A, Patil N, Chao M, Rodriguez-Boulan E (1991) An internal deletion in the cytoplasmic tail reverses the apical localization of human NGF receptor in transfected MDCK cells. J Cell Biol 115:607-618.

Levi-Montalcini R (1952) Effects of mouse tumor transplantation on the nervous system. Ann NY Acad Sci 55:330-343.

Levi-Montalcini R (1975) NGF: An unchartered route. In: The neurosciences: paths of discovery (Worden FG, Swazey JP, Adelman G, eds), pp 243-265. Cambridge, MIT.

Levi-Montalcini R (1976) The nerve growth factor: Its role in growth, differentiation, and function of the sympathetic adrenergic neuron. Prog Br Res 45: 235-258.

Levi-Montalcini R, Hamburger V (1951) A diffusible agent of mouse sarcoma producing hyperplasia of sympathetic ganglia and hyperneurotization of the chick embryo. J Exp Zool 123:321-362.

Levi-Montalcini R, Meyer H, Hamburger V (1954) *In vitro* experiments on the effects of mouse Sarcoma 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Caner Res 14:49-57.

Liu DT, Reid MT, Bridges DCM., Rush RA (1996) Denervation, but not decentralization, reduces nerve growth factor content of the mesenteric artery. J Neurochem. 66:2295-2299.

Loeb DM, Maragos J, Martin-Zanca D, Chao MV, Parada LF, Greene LA (1991) The *trk* protooncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell 66:961-966.

Loy R, Lachyankar MB, Condon PJ, Poluha DK, Ross AH (1994) Retrograde axonal transport and lesion-induced upregulation of the trkA high-affinity NGF receptor. Exp Neurol 130:377-386.

Lucidi-Phillipi CA, Clary DO, Reichardt LF, Gage FH (1996) TrkA activation is sufficient to rescue axotomized cholinergic neurons. Neuron 16:653-663.

Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than $p140^{trk}$ binding. J Biol Chem 269:6884-6891.

Martinou JC, Martinou I, Kato AC (1992) Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. Neuron 8:737-744.

Marty S, Peschanski M (1995) Effects of target deprivation on the morphology and survival of adult dorsal column nuclei neurons. J Comp Neurol 356:523-536.

Meakin SO, Shooter EM (1992) The nerve growth factor family of receptors. TINS 15:323-331.

Miller FDD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG (1994) Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. Dev Biol 161:206-217.

Modrell B, Liu J, Miller H, Shoyab M (1994) LIF and OM directly interact with a soluble form of gp130, the IL-6 receptor signal transducing subunit. Growth Factors 11:81-91.

Murphy M, Reid K, Hilton DJ, Bartlett PF (1991) Generation of sensory neurons is stimulated by leukemia inhibitory factor. Proc Natl Acad Sci USA 88:3498-3501.

Murphy M, Reid K, Brown MA, Bartlett PF (1993) Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. Development 117:1173-1182.

Nagata Y, Ando M, Takahama K, Iwata M, Hori S, Kato K (1987) Retrograde transport of endogenous nerve growth factor in superior cervical ganglion of adult rats. J Neurochem 49:296-302.

Oppenheim RW (1989) The neurotrophic theory and naturally occurring motoneuron death. TINS 12:252-255.

Patterson PH, Chun LLY (1977) The induction of acetylcholine synthesis in primary culturs of dissociated rat sympathetic neurons. Dev Biol 56:263-280.

Palmatier MA, Hartman BK, Johnson EM Jr (1984) Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. J Neurosci 4:751-756.

Paravicini U, Stoeckel K, Thoenen H (1975) Biological importance of retrograde axonal transport of nerve growth factor in adrenergic neurons. Br Res 84:279-291.

Purves D (1976) Effect of nerve growth factor on synaptic depression after axotomy. Nature 260:535-536.

Purves D, Lichtman JW (1985) Principles of Neural Development. Sunderland, MA: Sinauer Associates Inc.

Rabizadeh S, Bredesen DE (1994) Is $p75^{NGFR}$ involved in developmental neural cell death. Dev Neurosci 16:207-211.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Rao MS, Landis SC (1990) Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron 5:899-910.

Rao MS, Patterson PH, Landis SC (1992) Multiple cholinergic differentiation factors are present in footpad extracts. Develop. 116:731-744.

Rao MS, Sun Y, Escary JL, Perreau J, Tresser S, Patterson PH, Zigmond RE, Brulet P, Landis SC (1993) Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. Neuron 11:1175-1185.

Richardson PM, Riopelle RJ (1984) Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. J Neurosci 4:1683-1689.

Rodríguez-Tébar A, Dechant G, Gotz R, Barde YA (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Rodriguez-Tébar A, Dechant G, Gotz R, Barde YA (1992) Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J. 11:917-922.

Rush RA, Mayo R, Zettler C (1995) The regulation of nerve growth factor synthesis and delivery to peripheral neurons. Pharmac Ther 65:93-123.

Sadaat S, Sendtner M, Rohrer H (1989) Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. J Cell Biol 108:1807-1816.

Schotzinger RJ, Landis SC (1988) Cholinergic phenotype developed by noradrenergic sympathetic neurons after innervation of a novel cholinergic target in vivo. Nature 335:637-639.

Schotzinger RJ, Landis SC (1990) Acquisition of cholinergic and peptidergic properties by sympathetic innervtion of rat sweat glands requires interaction with normal target. Neuron 5:91-100.

Schotzinger RJ, Yin X, Landis SC (1994) Target determination of neurotransmitter phenotype in sympathetic neurons. J Neurobiol 25:620-639.

Schwab ME (1977) Ultrastructural localization of a nerve growth factor-horseradish peroxidase (NGF-HRP) coupling product after retrograde axonal transport in adrengeric neurons. Br Res 130:190-196.

Schwab ME, Thoenen H (1982) Retrograde axonal transport. In: Handbook of Neurochemistry. (Lajtha A, ed) pp 381-404. New York: Plenum Press.

Shelton DL, Reichardt LF (1984) Expression of the β-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc Natl Acad Sci USA 81:7951-7955.

Shelton DL, Reichardt LF (1986) Studies on the expresion of the β-nerve growth factor (NGF) gene in the central nervous system: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. Proc Natl Acad Sci USA 83:2714-2718.

Shields DC, Harmon DL, Nunez F, Whitehead AS (1995) The evolution of haematopoietic cytokine/receptor complexes. Cytokine 7:679-688.

Smeyne RJ, Llein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted trk-NGF receptor gene. Nature 368:246-249.

Stöckel K, Thoenen H (1975) Retrograde axonal transport of nerve growth factor: specificity and biological importance. Br Res 85:337-341.

Stöckel K, Paravicini U, Thoenen H (1974) Specificity of the retrograde axonal transport of nerve growth factor. Br Res 76:413-421.

Stöckel K, Schwab M, Thoenen H (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: A biochemical and morphological study. Br Res 89:1-14.

Stöckel K, Guroff G, Schwab M, Thoenen H (1976) The significance of retrograde axonal transport for the accumulation of systemically administered nerve growth factor (NGF) in the rat superior cervical ganglion. Br Res 109:271-284.

Svendsen CN, Cooper JD, Sofroniew MV (1991) Trophic factor effects on septal cholinergic neurons. Ann New York Acad Sci 640:91-94.

Taga T, Narazaki M, Yasukawa K, Saito T, Miki D, Hamaguchi M, Davis S, Shoyab M, Yancopoulos GD, Kishimoto T (1992) Functional inhibition of hematopoietic and neurotrophic cytokines by blocking the interleukin 6 signal transducer gp130. Proc Natl Acad Sci USA 89:10998-11001.

Taniuchi M, Johnson EM Jr (1985) Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directed against the rat nerve growth factor receptor. J Cell Biol 101:1100-1106.

Thaler CD, Suhr L, Ip N, Katz DM (1994) Leukemia inhibitory factor and neurotophins support overlapping populations of rat nodose sensory neurons in culture. Dev Biol 161:338-344.

Thoenen H, Barde Y-A (1980) Physiology of nerve growth factor. Physiol Rev 60:1284-1334.

Thoenen H, Bandtlow C, Heumann R (1987) The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. Rev Physiol Biochem Pharm 109:146-178.

Tomac A, Widenfalk J, Lin LF, Kohno T, Ebendal T, Hoffer BJ, Olson L (1995) Retrograde axonal transport of glial cell line-derived neurotorphic factor in the adult nigrostriatal system suggests a trophic role in the adult. Proc Natl Acad Sci USA 92:8274-8278.

Verdi JM, Ip N, Yancopoulos GD, Anderson DJ (1994a) Expression of trk in MAH cells lacking the p75 low-affinity nerve growth factor receptor is sufficient to permit nerve growth factor-induced differentiation to postmitotic neurons. Proc Natl Acad Sci USA 91:3949-3953.

Verdi JM, Birren SJ, Ibánez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994b) $p75^{LNGFR}$ regulates trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron 12:733-745.

Vogel KS (1993) Development of trophic interactions in the vertebrate peripheral nervous system. Mol Neurobiol 7:363-382.

Von Bartheld CS, Williams R, Lefcort F, Clary DO, Reichardt LF, Bothwell M (1996) Retrograde transport of neurotrophins from the eye to the brain in chick embryos: Roles of the $p75^{NTR}$ and trkB receptors. J Neurosci 16:2995-3008.

Wayne DB, Heaton MB (1988) Retrograde transport of NGF by early chick embryo spinal cord motoneurons. Dev Biol 127:220-223.

Wheeler EF, Bothwell M (1992) Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. J Neurosci 12:930-945.

Whitemore SR, Ebendal T, Larkfors L, Olson L, Seiger A, Strömberg I, Persson H (1986) Developmental and regional expression of β-nerve growth factor messenger RNA and protein in the rat central nervous system. Proc Natl Acad Sci USA 83:817-821.

Wong V, Kessler JA (1987) Solubilization of a membrane factor that stimulates levels of substance P and choline acetyltransferase in sympathetic neurons. Proc Natl Acad Sci USA 84:8726-8729.

Yamamori T, Sarai A (1994) Evolution of the IL-6/class IB cytokine receptor family in the immune and nervous systems. J Physiol 88:165-171.

Yamamori Y, Fukada K, Ruedi A, Korsching S, Ming-Ji F, Patterson PH (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science 246:1412-1416.

Yan Q, Snider WD, Pinzone JJ, Johnson EM Jr (1988) Retrograde transport of nerve growth factor (NGF) in motoneurons of developing rats: Assessment of potential neurotrophic effects. Neuron 1:335-343.

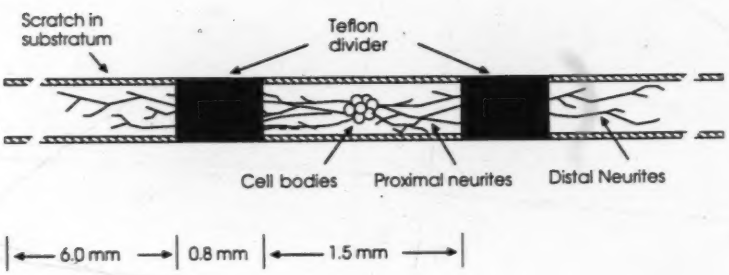
Zettler C, Zhou XF, Bridges D, Rush RA (1996) Increased concentrations of nerve growth factor detected in tissue extracts with an improved extraction procedure. J Neurosci Res (in press).

FIGURES

Figure 1.1 Compartmented culture design

The illustration above shows the placement of a Teflon divider in a collagen-coated culture dish in which parallel scratches in the substrate have been made. The illustration below shows neurons along 1 of the 20 collagen tracks from a typical culture.

Teflon divider
Side Compartment
35 mm tissue culture dish
Center compartment


Scratch in substratum
Teflon divider
Cell bodies
Proximal neurites
Distal Neurites
6.0 mm
0.8 mm
1.5 mm

Chapter 2

Cholinergic Differentiation of Rat Sympathetic Neurons in Culture: Effects of Factors Applied to Distal Neurites

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INTRODUCTION

A well characterized model of target regulation of neuronal phenotype is the cholinergic switch. First described in cultured rat sympathetic neurons (Patterson and Chun, 1977), it involves a reduction in catecholaminergic properties and a simultaneous induction of cholinergic properties in response to a variety of externally applied factors and conditions (see Landis, 1990, for review). Changes in neuropeptide levels have also been described (Kessler, 1985; Nawa and Patterson, 1990; Nawa and Sah, 1990; Rao and Landis, 1990).

Experiments on the innervation of the sweat glands in the foot pad of the rat suggest that cholinergic switching factors released by the target tissue can alter the transmitter phenotype of the innervating sympathetic neurons (Schotzinger and Landis, 1988). At birth, sympathetic neurons innervating the sweat glands synthesize and store catecholamines and lack the ability to synthesize and store acetylcholine. The cholinergic switch begins at about postnatal day 11 when choline acetyltransferase (ChAT) first becomes detectable and progresses until, in the adult, the innervating neurons have become functionally cholinergic. When sweat glands from neonatal rats are transplanted to a region of hairy skin, innervating sympathetic neurons which normally remain noradrenergic become cholinergic. Conversely, if the sweat glands of early postnatal rats are removed and the foot pads replaced with parotid glands which normally receive noradrenergic innervation, the innervating neurons remain noradrenergic (Schotzinger and Landis, 1990). Sweat gland extract given to sympathetic neurons in culture also induces the cholinergic switch (Rao and Landis, 1990). These experiments suggest that cholinergic switching is induced by target-derived factor(s) at axon terminals.

Several purified factors, as well as media conditioned by a variety of cultured tissues, when applied to sympathetic neurons in mass cultures, induce cholinergic switching. Some of the purified factors include leukemia inhibitory factor (LIF), which is identical to cholinergic

differentiation factor (CDF; Weber, 1981; Fukada, 1985; Yamamori et al., 1989), 2 spinal cord membrane-associated factors (Wong and Kessler, 1987; Adler et al., 1989), ciliary neurotrophic factor (CNTF; Saadat et al., 1989), and rat sweat gland extract (Rao and Landis, 1990). To address the question of whether neurons are capable of responding to switching factors applied only to axon terminals we used compartmented cultures in which distal neurites are spatially separated from cell bodies and proximal neurites. Our results demonstrate that recombinant LIF/CDF, heart cell conditioned medium, and dermal fibroblast conditioned medium, when supplied only to distal neurites, induce the cholinergic switch in cultured rat sympathetic neurons.

MATERIALS AND METHODS

Culture Procedures

Superior cervical ganglia were dissected from newborn Sprague-Dawley rat pups (supplied by University of Alberta Farm). The ganglia were chemically dissociated by separate incubations in 0.1% trypsin (Calbiochem, La Jolla, CA) and 10 μg/ml DNAase (Sigma, St. Louis, MO), followed by mechanical dissociation as previously described (Campenot et al., 1991). General culture procedures essentially followed the methods of Hawrot and Patterson (1979). The standard culture medium was L15CO2 medium (GIBCO Laboratories, Grand Island, NY) supplemented with the prescribed additives and 6% methylcellulose.

Compartmented cultures were constructed as previously described (Campenot, 1979, 1982). Rat tail collagen was prepared and air-dried onto 35 mm tissue culture dishes (Hawrot and Patterson, 1979). Parallel collagen tracks were made by scratching the collagen substrate with a lab-made pin rake. A Teflon divider (Tyler Research Instruments, Edmonton, Alberta, Canada) was then seated on top of the tracks.

Approximately 2500 (single density) or 5000 (double density) cells were plated in the center compartment of each dish. The center-compartment medium consisted of standard L15CO2 supplemented with 200 ng/ml 2.5S nerve growth factor (NGF; kindly supplied by Dr. Richard Murphy), 2.5% rat serum (prepared by Lab Animal Services, University of Alberta), 1 mg/ml ascorbic acid, and 10 μM cytosine arabinoside (Sigma) to limit division of nonneuronal cells. Neurites were allowed to grow into the two target (side) compartments by supplementing L15CO2 medium in the target compartments with 200 ng/ml NGF. After 5-7 days when many neurites had crossed the silicon grease barriers and for all further medium changes, fresh medium containing 2.5% rat serum and 1 mg/ml ascorbic acid but lacking NGF and cytosine arabinoside was given to the center compartments. Target compartments were supplied with 200 ng/ml NGF throughout the experiments. All cultures were incubated in a 5% CO_2 atmosphere at 37°C, and medium was changed every 3-4 days.

Treatments

The treatments consisted of bathing distal neurites (side compartments) with treatment media. Recombinant human LIF expressed in E. coli (Mr 20 kD) was kindly provided by Dr. Larry Guilbert (University of Alberta). The stock concentration was 80,000 units/ml as measured by the DA1.a cell line proliferation assay. The activity of hyperglycosylated recombinant human LIF (Mr 100 KDa) has been reported to be ≈ 20 units/ng (Gearing et al., 1991), which is similar to nonglycosylated LIF (Gough et al., 1988). Purified recombinant human LIF and anti-LIF polyclonal IgG were purchased from R&D Systems Inc. (Minneapolis, MN).

Heart cell conditioned medium was made from neonatal rat hearts. After mincing 5 hearts, the cells (myocytes and fibroblasts) were dissociated by gentle stirring of the pieces in a physiological saline (L15 inorganic salts recipe) containing 0.5 mg/ml collagenase (Sigma). Dissociated cells were collected and rinsed in L15-CO2 containing 10% fetal calf serum. L15-CO2 with 2.5% rat serum was used for the final cell suspension and all further medium changes. The cells were plated in 75 cm^2 tissue culture flasks. Upon reaching near confluency, the cultures were incubated in fresh medium, usually two days, after which time the conditioned medium was collected and pooled. Conditioning in the same cultures was repeated until the cells began to detach from the flask surface.

Rat dermal fibroblasts were obtained from neonatal rat skin. Pieces of dermis were scraped from skin fragments and incubated in aliquots of 0.25% trypsin/0.25% collagenase and then triturated to obtain dissociated cells. These were rinsed in DMEM/10% fetal calf serum, filtered through sterile mesh, and resuspended in the same medium type. After the cells grew near confluence in 75 cm^2 flasks, the medium was replaced with L15-CO2 without serum. The medium was conditioned for 4 days and the aliquots from the flasks were pooled.

In all experiments conditioned media were diluted to 50% with L15-CO2. To control for the presence of 2.5% rat serum in the medium conditioned by heart cells, rat serum was added to the fresh diluting medium, as well as to DFCM and LIF treatment media to maintain a final level of 2.5%. NGF was added to a final concentration of 200 ng/ml.

Assays

Sympathetic neurons were harvested from the culture dishes for measurement of total protein and choline acetyltransferase activity and semi-quantitation of tyrosine hydroxylase levels. Media was removed and the cultures were rinsed twice with cold phosphate buffered saline (PBS). After removal of the Teflon divider, the cells were gently pipetted off the dish in ice-cold PBS. All the cells from the same treatment were pooled and pelleted at 1160 RPM. The pellet was resuspended in PBS and transferred to a microcentrifuge tube. After a 30 second spin the supernatant was replaced with cold ChAT homogenization buffer (0.5% Triton X-100 in sodium phosphate buffer, pH 6.8) at a volume corresponding to approximately 20 µl/culture, the tissue was homogenized by micropestle, vortexed, and frozen. Except during centrifugation, the samples were always kept on ice. Prior to analysis, the solution was thawed, and undissolved material was pelleted by centrifugation and discarded.

A 20 µl aliquot of the dissolved tissue from each treatment pool was assayed colorimetrically for total protein content using the micro version of the Peterson protein assay (Peterson, 1977), with normal mouse IgG as the standard. The reaction product was measured spectrophotometrically at an optical density of 750 nm. Protein levels in the sample aliquots were calculated by regression analysis based on the protein standards.

Choline acetyltransferase activity in 20 µl aliquots from the same treatment pools of dissolved tissue was measured using the modified version of the assay by Fonnum (1975). The initial acetyl-CoA concentration was 135 µM, and incubations were performed for 30 or 40 minutes. Experiments showed that this variation in incubation time did not change the specific enzyme activity. These assays permitted the calculation of specific ChAT activity in pmol of product/min•mg of protein, where the product represented acetylcholine. LIF- and HCCM-induced ChAT activity was inhibited by an average of 93% in the presence of the specific ChAT inhibitor, napthylvinyl pyridine (0.5 mM), demonstrating that the large majority of reaction product in the ChAT assay was the result of activity by choline acetyltransferase and not by the closely related enzyme, carnitine acetyltransferase (Patterson et al., 1975).

The levels of tyrosine hydroxylase were semi-quantified by Western blot analysis. Volumes of sample material adjusted to achieve equal protein were loaded on 10% sodium

dodecyl sulfate-polyacrylamide minigels (SDS-PAGE). Protein equivalency was verified by silver-staining duplicate gels. Proteins were blotted onto nitrocellulose, and nonspecific binding was blocked by incubation in 5% skim milk powder with 0.3% Tween 20. The primary antibody was a monoclonal mouse anti-TH whole IgG, as reported previously (Rohrer et al., 1986), while the secondary antibody was alkaline-phosphatase conjugated goat anti-mouse IgG (Boehringer Mannheim). The reaction product was developed with Nitro Blue Tetrazolium (Sigma) and 5-bromo-4-chloro-3-indoyl phosphate (Sigma) in 10 mM bicarbonate buffer. The alkaline phosphatase signals were densitometrically analyzed by processing a video camera image of the blot using MacIntosh Image 1.37 software.

RESULTS

Choline Acetyltransferase Acivity Induced By Factors On Distal Neurites

Neurons dissociated from superior cervical ganglia of newborn rats were plated in the center compartments of 3-compartment cultures. They were maintained throughout the experiments with 2.5% rat serum in all compartments. During the first 7 days 200 ng/ml 2.5S NGF was supplied to all compartments, and neurites extended into the left and right target compartments, thus allowing manipulation of the fluid environment of distal neurites separately from that of cell bodies and proximal neurites. After 7 days, cultures were divided into 3 treatment groups such that one of the following treatment media was supplied to only distal neurites in left and right target compartments: 600 units/ml recombinant (human) LIF; 50% rat heart cell conditioned medium (HCCM), or; 50% rat dermal fibroblast conditioned medium (DFCM). Control cultures were given identical medium in left and right target compartments but without cholinergic switching factors. During the treatment period all cultures were supplied with 200 ng/ml NGF only in the target compartments. After 14 days of treatment the dividers were removed and the neurons from each treatment group were pooled. Aliquots from each pool were assayed for total protein by the Peterson method and ChAT activity by the Fonnum method (see Materials and Methods). Each culture dish contained about 6 μg of protein and this did not differ between control and LIF treatment groups (Wilcoxon Signed-Rank Test, $p=0.01$). Furthermore, when the same harvesting steps and protein assay were performed on culture dishes lacking cells, protein derived from residual serum and the collagen substrate accounted for less than 5% of the total protein levels of neuronal cultures.

Treatment of distal neurites with LIF, HCCM, or DFCM significantly induced ChAT activity over untreated controls (Figure 2.1). This was apparent as a minimum 6.5-fold increase in cpm values of treated samples over untreated samples when equal volumes of homogenized tissue were assayed. When the ChAT activity calculated from cpm values was normalized to protein content, the specific ChAT activity of tissue samples treated with switching factors ranged from 132.1 - 1050.7 pmol/min•mg. The largest average induction occurred after LIF treatment, corresponding to 720.4 pmol/min•mg. ChAT activity in untreated neuron samples

could also be calculated, but the cpm values from these aliquots were only marginally higher than cpm from concurrently-run ChAT buffer blanks. Eight of the 9 trials on untreated samples did not surpass our arbitrary threshold (2X blank cpm) representing specific ChAT activity, and we therefore considered control ChAT activity to be negligible. The single qualifying trial of an untreated sample gave a cpm value that was 2.3X higher than that of the blank, which corresponded to a specific ChAT activity of 35.7 pmol/min•mg. Thus, there was no overlap in specific ChAT activity between any of the treated and untreated tissue samples. The absolute values of specific ChAT activity we obtained were similar to values reported previously (Patterson and Chun, 1977).

In 2 platings (shown in Figure 2.1) the plating density of neurons was doubled. Even though increased neuron density in mass cultures has been shown to enhance the cholinergic switch (Adler and Black, 1985; Kessler, 1985), no trend in our data towards higher ChAT activity in double density cultures was observed. Since the cell bodies in the center compartment aggregated at both plating densities, there may not have been differences in cell contact sufficient to produce significant changes in ChAT activity between the 2 types of cultures.

The time course of ChAT activity induction by LIF was investigated by treatment of 7 day-old cultures (designated treatment day 0). LIF at a concentration of 300 units/ml, along with 200 ng/ml NGF and 2.5% rat serum, was supplied to the target compartments for up to 14 days. After 0, 3, 7, 10, and 14 days of treatment, groups of 3 cultures were harvested and pooled, and specific ChAT activities calculated from the homogenates. The activity in treatment day 0 neurons was considered too low to be significant. Observable activities at later time points suggested a trend of increasing cholinergic induction throughout the 14 day treatment period. The maximum ChAT activity observed was 923.3 pmol/min•mg (Figure 2.2).

The concentration-dependence of LIF-mediated ChAT induction was studied by once again applying LIF to target compartments of 7-day-old cultures. Rat serum and NGF was present in the target compartments as in previous experiments. Distal neurites were supplied with control medium or medium containing various concentrations of LIF for the next 7 days, a treatment period previously found to give substantial and reproducible inductions in ChAT activity. Neurons from 2 or 3 cultures from each treatment were then pooled and assayed. At concentrations up to 10 units/ml LIF there was either no or very low levels of specific ChAT activity, but at higher concentrations considerable induction of activity occurred (Figure 2.3).

The maximum specific activity occurred at the highest concentration tested, 1000 units/ml LIF, and was equivalent to 377.8 pmol/min•mg. However, the large variability in ChAT activity within treatments precluded a firm determination of the shape of the concentration-effect curve.

Cholinergicity could also be induced by applying switching factors to only the center compartment or to all three compartments. In one experiment in which 600 units/ml LIF was applied to cultures for 9 days, ChAT activities were 274.8 pmol/min•mg when LIF was applied to the side compartments and 405.6 pmol/min•mg (1.5-fold higher) when LIF was applied to only the center compartments. In another experiment in which 300 units/ml LIF was applied for 14 days to side compartments, the ChAT activity was 484.0 pmol/min•mg, but it doubled to 965.5 pmol/min•mg in other cultures when LIF was applied to all 3 compartments. Similarly, the ChAT activity of neurons that had been treated for 14 days with 50% HCCM in the side compartments was 277.1 pmol/min•mg but it increased to 648.6 when all 3 compartments were treated. The results of the above 3 experiments are representative of observations from other experiments in which treatment conditions varied slightly (data not shown). The observation that ChAT activity was induced to slightly higher levels in cultures that received LIF in the center compartments compared to cultures that received LIF in target compartments may simply indicate a relationship between cholinergic function and the amount of neuronal surface exposed to factor. It should be remembered that no firm conclusions can be made about the LIF responsiveness of neurites relative to cell bodies because neurites are also present in the center compartment.

To verify that ChAT activity could be induced by the presence of switching factors on distal neurites alone, it was necessary to confirm that there was no leakage of switching factors from the side compartments into the center compartment. A variety of experiments have revealed that the leakage between compartments is negligible. Firstly, no bulk flow of medium occurs between adjacent compartments filled to different levels, despite the hydrostatic fluid pressure (Campenot, 1979). At a molecular level, atomic emission spectroscopy of calcium levels has shown that some transfer of free calcium occurs between compartments but at a very slow rate. A 100-fold free Ca^{2+} gradient of 1.0 mM:10 μM is reduced by only 1.4-1.7 μM/day (Campenot and Draker, 1989). It has also been shown that virtually no radiolabelled choline crosses from one compartment into another over a 15 hr time period (Vance et al., 1991). In the present experiments we used a polyclonal antibody to recombinant human LIF to block the biological

activity of the purified factor. 5 μg/ml of anti-LIF antibody applied concurrently with 2.5 ng/ml of LIF to the target compartments for 8 days completely blocked the induction of ChAT activity by LIF, as measured from 2 pools of neuron homogenate (3 cultures each). When the same concentration of antibody was applied for 8 days to the center compartments of cultures whose target compartments also received 2.5 ng/ml LIF, the observed ChAT activity from 2 pools of neuron homogenate (3 cultures each) was 945.7 ± 88.6 (SEM) pmol/min•mg. Since center-applied anti-LIF would have neutralized any LIF that had leaked from target compartments to center compartments, the cholinergic induction by distally-applied LIF must have been entirely the result of LIF in target compartments.

Tyrosine Hydroxylase Levels Reduced By Factors On Distal Neurites

LIF and conditioned media treatment of distal neurites not only increased specific ChAT activity but also decreased the levels of tyrosine hydroxylase, the rate-limiting enzyme involved in catecholamine synthesis (Figure 2.4). Target compartments were supplied with 600 units/ml LIF, 50% HCCM, or 50% DFCM, along with rat serum and NGF, from Days 7-21 in culture. Neurons from several cultures were then harvested and pooled. After first quantifying protein levels in each of the pooled samples, volumes of sample containing equal protein were run on SDS-PAGE and blotted onto nitrocellulose. Equivalent protein levels were confirmed by silver-staining separately run SDS polyacrylamide gels. Immunoblotting with a monoclonal antibody to TH revealed a reduction in the expression of TH, which was quantified by profiling the density of the alkaline phosphatase signal using a computer-assisted image processing system. After treating distal neurites for 2 weeks the average reductions (± SEM) in TH levels for multiple experiments were 68 ± 13% (N=3), 61 ± 11% (N=4), and 74 ± 9% (N=3) for 600 unit/ml LIF, 50% HCCM, and 50% DFCM treatments, respectively.

Cholinergic Switching Factors Induce Changes In Neurite Morphology

The cholinergic switch induced by LIF and conditioned media on distal neurites was correlated with the morphology of the neurites in the target compartments (Figure 2.5). After 21 days in culture neurites of untreated cultures covered a large portion of the surface area of each

collagen track (Panel *a*). In addition they were often fasciculated, forming thick cables which ran the length of the tracks. Neurites that had been treated with LIF (Panel *b*) or conditioned media (Panel *c,d*) from days 7-21 in culture were more concentrated into a central cable and covered far less of the collagen surface than control neurites. Although rat serum can cause an increase in cabled morphology (Campenot, 1982), both control and treatment cultures were maintained in 2.5% rat serum, thus excluding the possibility that serum alone could account for the increased fasciculation of treated neurites. The increased fasciculation in all treatment groups suggest that cholinergic switching factors might have additional effects beyond the regulation of neurotransmitter phenotype.

DISCUSSION

Induction by target tissues represents an effective mechanism by which neurons can acquire specific phenotypes. The concept as applied to sympathetic neurons is well supported by transplantation experiments involving rat sweat glands. One premise of the theory of target-mediated induction is that inducing factors can exert their effect when present only at distal endings of nerve fibers. Based on this theory, our investigations support the idea that LIF and factors in heart cell and dermal fibroblast conditioned media, if directly available to sympathetic neuron terminals, could exert such a distally-mediated effect on the cholinergic switch in vivo.

Exposure of distal neurites in target compartments to switching factors was sufficient to induce ChAT activity in the neurons, although the induction approximately doubled when the factors were applied to all compartments. It is difficult to assess the meaning of this information in terms of receptor distribution on neurites and cell bodies for two reasons. Firstly, the presence of both cell bodies and neurites in the center compartment makes it impossible to attribute observed activities to one or the other of these cell structures. Secondly, there is no data on the relative cell surface areas in the target compartments versus the center compartment. A clear analysis of receptor distribution awaits experiments using labelled switching factors.

LIF is identical to CDF (Yamamori et al., 1989). CDF was the first cholinergic-inducing factor purified, being isolated from rat heart cell conditioned medium (Fukada, 1985). LIF has been implicated in nonneuronal systems where its effects are extremely diverse, affecting differentiation, proliferation, and/or metabolism both negatively or positively, depending on the cell type (for reviews, see Hilton and Gough, 1991; Kurzrock et al., 1991). LIF has previously been shown to maintain the survival of mouse sensory neurons (Murphy et al., 1991) and to induce the cholinergic switch in mass cultures of rat sympathetic neurons (Yamamori et al., 1989).

Others have shown that medium conditioned by cultured neonatal rat skin induces the cholinergic switch (Geiss and Weber, 1984; Nawa and Sah, 1990). It is not known whether the switching factor in rat dermal fibroblast conditioned medium is also LIF, but there is circumstantial evidence suggesting this. Fibroblasts are a likely candidate for producing the switching factor in heart cell cultures and are a common component of the majority of mixed cultures which produce cholinergic switching activity. Also primary and immortalized

fibroblasts in culture produce switching activity (Patterson and Chun, 1977). Regardless of the nature of the DFCM switching factor, it is interesting that neonatal dermal fibroblasts in culture secrete a switching factor, since hairy skin in the rat, from which dermal fibroblasts are derived, is normally innervated by noradrenergic (i.e. "unswitched") neurons (Schotzinger and Landis, 1988).

A close correlation was observed between the switching event and the fasciculated morphology of the neurites. Although the phenomenon could be interpreted in a variety of ways, the neurite morphology does resemble a relative shift in the adhesion affinity towards a neurite-neurite interaction rather than a neurite-substrate interaction. It has been shown that surface molecule characteristics of rat sympathetic neurons change concurrent with a switch to a cholinergic phenotype, but it is unknown whether these changes involve alterations in adhesion phenomena. The neurotransmitter switch has been shown to increase the expression of one surface glycoprotein, while decreasing the expression of another (Braun et al., 1981). In addition the binding of soybean agglutinin (SBA) to surface membrane increases with age of adrenergic neurons but not cholinergic neurons, while the binding of cholera toxin increases as cholinergic neurons age but decreases as adrenergic cultures age (Schwab and Landis, 1981). The decreased binding of SBA to cholinergic neurons reflects lesser amounts or accessibility of the lectin to two neutral glycolipids on the cell surface, GL-4 and GL-X (Zurn, 1982). A number of gangliosides are also known to differ quantitatively between adrenergic and cholinergic neurons (Zurn, 1982). In chick sympathetic neurons the cell contact-mediated up-regulation of ChAT activity requires NCAM homophilic binding, but does not occur if the NCAM present on the membranes is heavily polysialylated (Acheson and Rutishauser, 1988). If the application of cholinergic switching factors affects adhesion processes, the possibility exists that cholinergic differentiation is at least in part the result of an increase in cell contact, since increasing sympathetic neuron cell density (Adler and Black, 1985) or addition of isolated membranes from ganglionic nonneuronal cells or sympathetic, sensory, or spinal cord neurons (Kessler et al., 1986; Wong and Kessler, 1987; Adler et al., 1989) also induce ChAT activity. Further elucidation of the identities and functions of the phenotype-specific surface molecules could possibly lend insight into the reasons for the dichotomous neurite morphologies.

Given that cell membranes and conditioned medium from a variety of common cell types induce the cholinergic switch, it is surprising that cholinergic switching is uncommon in vivo.

Our finding that distally-supplied LIF and conditioned media induce cholinergic switching suggests that the rarity of switching in vivo is likely not due to an inability of sympathetic neurons to respond to these factors in the periphery. Sympathetic neurons in culture clearly possess the mechanism to translate a chemical signal in peripheral regions into biochemical and morphological changes in the cells. Only one other biological molecule, nerve growth factor, has so far been shown to exert pleiotropic effects when applied to distal neurites in culture (Campenot, 1982). A wide variety of differentiative effects have been attributed to both NGF and LIF, and it will be interesting to determine the extent to which the signal translation machinery is shared by these 2 biochemical cues.

REFERENCES

Acheson, A., and Rutishauser, U. (1988). Neural cell adhesion molecule regulates cell contact-mediated changes in choline acetyltransferase activity of embryonic chick sympathetic neurons. J. Cell Biol. 106, 479-486.

Adler, J. E., and Black, I. B. (1985). Sympathetic neuron density differentially regulates transmitter phenotype expression in culture. Proc. Natl. Acad. Sci. USA 82, 4296-4300.

Adler, J. E., Schleifer, L. S., and Black, I. B. (1989). Partial purification and characterization of a membrane-derived factor regulating neurotransmitter phenotypic expression. Proc. Natl. Acad. Sci. USA 86, 1080-1083.

Campenot, R. B. (1979). Independent control of the local environment of somas and neurites. Meth. Enzymol. 28, 302-307.

Campenot, R. B. (1982). Development of sympathetic neurons in compartmentalized cultures I. Local control of neurite growth by nerve growth factor. Dev. Biol. 93, 1-12.

Campenot, R. B., and Draker, D. D. (1989). Growth of sympathetic nerve fibers in culture does not require extracellular calcium. Neuron 3, 733-743.

Campenot, R. B., Walji, A. H., and Draker, D. D. (1991). Effects of sphingosine, staurosporine, and phorbol ester on neurites of rat sympathetic neurons growing in compartmented cultures. J. Neurosci. 11, 1126-1139.

Fonnum, F. (1975). A rapid radiochemical method for the determination of choline acetyltransferase. J. Neurochem. 24, 407-409.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc. Natl. Acad. Sci. USA 82, 8795-8799.

Gearing, D. P., Thut, C. J., VandenBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D., and Beckmann, M. P. (1991). Leukemia inhibitory factor is structurally related to the IL-6 signal transducer, gp130. EMBO J. 10: 2839-2848.

Geiss, M.-C., and Weber, M. J. (1984). Acetylcholine metabolism in rat spinal cord cultures: Regulation by a factor involved in the determination of the neurotransmitter phenotype of sympathetic neurons. J. Neurosci. 4, 1442-1452.

Gough, N. M., Gearing, D. P., King, J. A., Willson, T., Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988). Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. Proc. Natl. Acad. Sci. 85: 2623-2627.

Hawrot, E., and Patterson, P. H. (1979). Long-term cultures of dissociated sympathetic neurons. Meth. Enzymol. 28, 574-584.

Hilton, D. J., and Gough, N. M. (1991). Leukemia inhibitory factor: A biological perspective. J Cell. Biochem. 46, 21-26.

Kessler, J. A. (1985). Differential regulation of peptide and catecholamine characters in cultured sympathetic neurons. Neurosci. 15, 827-839.

Kessler, J. A., Conn, G., and Hatcher, V. B. (1986). Isolated plasma membranes regulate transmitter expression and facilitate effects of a soluble brain cholinergic factor. Proc. Natl. Acad. Sci. USA 83, 3528-3532.

Kurzrock, R., Estrov, Z., Wetzler, M., Gutterman, and Talpaz, M. (1991). LIF: Not just a leukemia inhibitory factor. Endocrine Rev. 12, 208-216.

Landis, S. C. (1990). Target regulation of neurotransmitter phenotype. TINS 13, 344-350.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. Proc. Natl. Acad. Sci. USA 88, 3498-3501.

Nawa, H., and Patterson, P. H. (1990). Separation and partial characterization of neuropeptide-inducing factors in heart cell conditioned medium. Neuron 4, 269-277.

Nawa, H., and Sah, D. W. Y. (1990). Different biological activities in conditioned media control the expression of a variety of neuropeptides in cultured sympathetic neurons. Neuron 4, 279-287.

Patterson, P. H., and Chun, L. L. Y. (1977). The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. Dev. Biol. 56, 263-280.

Patterson, P. H., Reichardt, L. F., and Chun, L. L. Y. (1975). Biochemical studies on the development of primary sympathetic neurons in cell culture. Cold Spring Harbor Symp. Quant. Biol. 40, 389-397.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346-356.

Rao, M. S., and Landis, S. C. (1990). Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron 5, 899-910.

Rohrer, H., Acheson, A. L., Thibault, J., and Thoenen, H. (1986). Developmental potential of quail dorsal root ganglion cells analyzed in vitro and in vivo. J. Neurosci. 6, 2616-2624.

Schotzinger, R. J., and Landis, S. C. (1988). Cholinergic phenotype developed by noradrenergic sympathetic neurons after innervation of a novel cholinergic target in vivo. Nature 335, 637-639.

Schotzinger, R. J., and Landis, S. C. (1990). Acquisition of cholinergic and peptidergic properties by sympathetic innervation of rat sweat glands requires interaction with normal target. Neuron 5, 91-100.

Sussdorf, W. S., and Campenot, R. B. (1986). Influence of the extracellular potassium environment on neurite growth in sensory neurons, spinal cord neurons and sympathetic neurons. Dev. Br. Res. 25: 43-52.

Vance, J. E., Pan, D., Vance, D. E., and Campenot, R. B. (1991). Biosynthesis of membrane lipids in rat axons. J. Cell Biol. 115, 1061-1068.

Weber, M. J. (1981). A diffusible factor responsible for the determination of cholinergic functions in cultured sympathetic neurons. J. Biol. Chem. 256, 3447-3453.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336, 684-687.

Wong, V., and Kessler, J. A. (1987). Solubilization of a membrane factor that stimulates levels of substance P and choline acetyltransferase in sympathetic neurons. Proc. Natl. Acad. Sci. USA 84, 8726-8729.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science 246, 1412-1416.

FIGURES

Figure 2.1 Induction of choline acetyltransferase by factors applied to distal neurites

Target compartments containing neurites of sympathetic neurons were supplied with medium containing 200 ng/ml NGF and either LIF (600 units/ml), 50% heart cell conditioned medium, or 50% dermal fibroblast conditioned medium from days 7-21 in culture. Neurons from 2-10 cultures from separate treatments were then pooled, and aliquots were assayed for ChAT activity and total protein. Data are expressed as absolute specific ChAT activity. Each symbol represents the results of an individual trial, and alike symbols represent trials from the same plating of neurons. Filled symbols represent experiments with double density cultures.

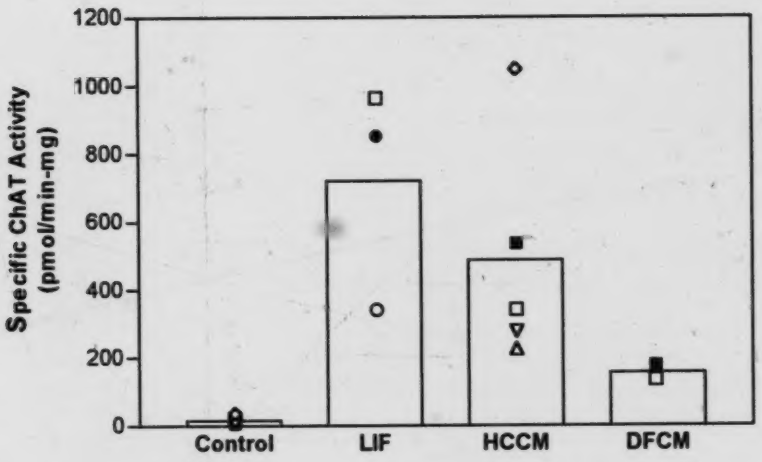

Specific ChAT Activity
(pmol/min-mg)
1200
1000
800
600
400
200
0
Control
LIF
HCCM
DFCM

Figure 2.2 Time dependence of induction of specific ChAT activity by LIF

Sympathetic neurons were allowed to grow for 7 days, at which time (treatment day 0) distal neurites in target compartments were supplied with 200 ng/ml NGF and 300 units/ml LIF for up to 14 days. For each trial neurons from 3 cultures were pooled and equal aliquots assayed for ChAT activity and total protein. Data are expressed as absolute specific ChAT activity. Each symbol represents an individual trial, and alike symbols represent trials from the same plating of neurons. A third order regression of the mean activities is plotted showing the time-dependent trend.

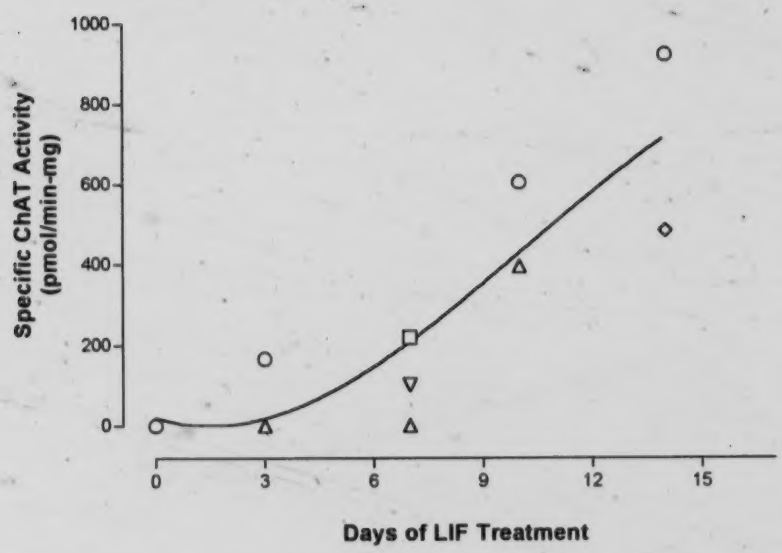

Specific ChAT Activity
(pmol/min-mg)
1000
800
600
400
200
0
0
3
6
9
12
15
Days of LIF Treatment

Figure 2.3 Concentration dependence of induction of specific ChAT activity by LIF

Sympathetic neurons were allowed to grow for 7 days, after which time neurites in target compartments were supplied 200 ng/ml NGF and LIF at the indicated concentration for 7 days. For each trial neurons from 2-3 cultures were pooled and assayed for total protein and ChAT activity. Data are expressed as absolute specific ChAT activity. Each symbol represents an individual trial, and alike symbols represent trials from the same plating of neurons. A third order regression of the mean activities is plotted showing the concentration-dependent trend.

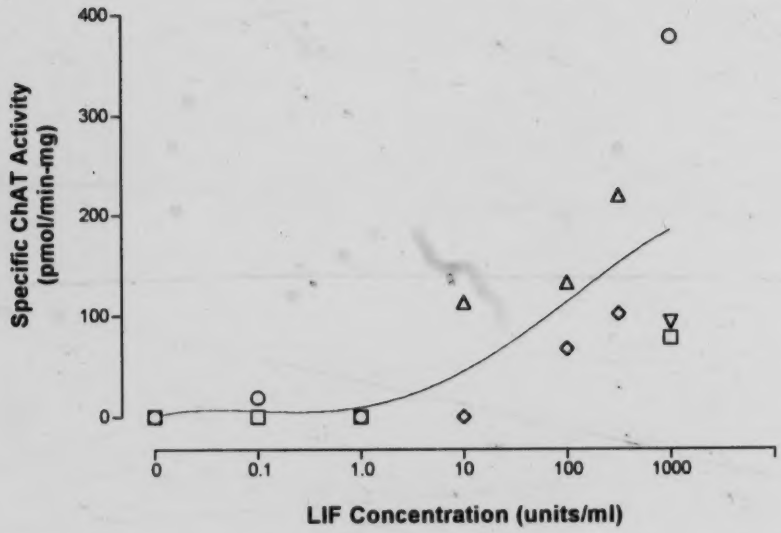
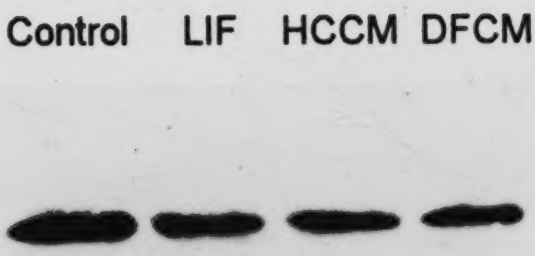

Specific ChAT Activity
(pmol/min-mg)
400
300
200
100
0
0
0.1
1.0
10
100
1000
LIF Concentration (units/ml)

Figure 2.4 Reduction in tyrosine hydroxylase by factors applied to distal neurites

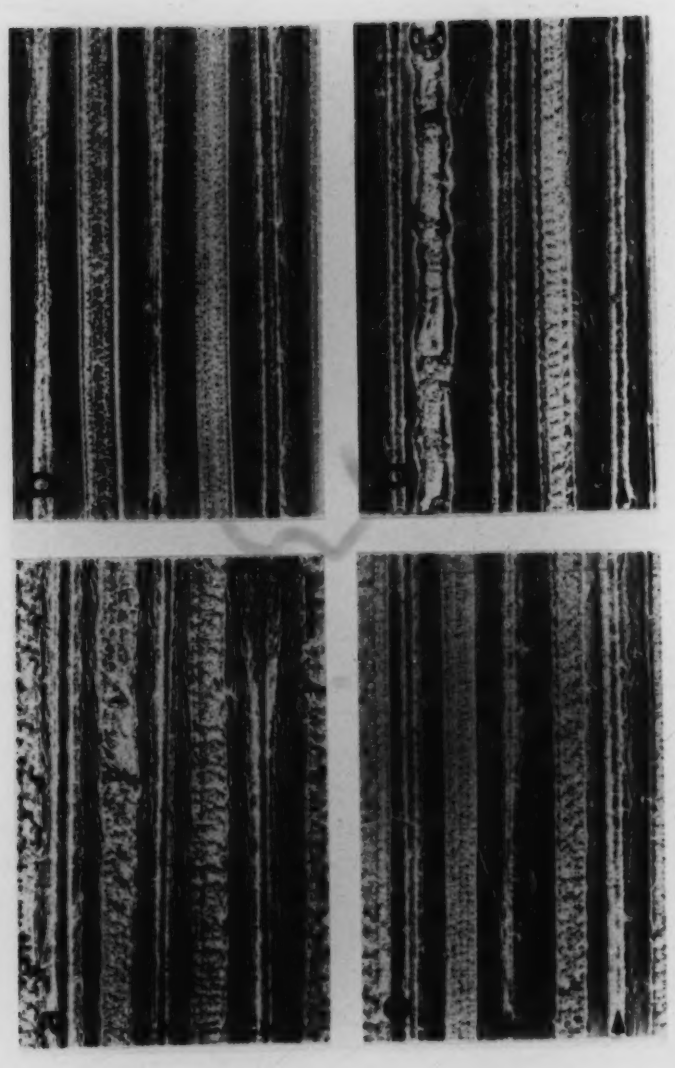
Sympathetic neurons were treated as in Fig. 2.1. Protein-equivalent aliquots from each treatment were run on SDS-PAGE and blotted onto nitrocellulose. Blots were probed with a monoclonal antibody to TH and secondarily with an alkaline phosphatase-coupled secondary antibody. TH signal density was analyzed using an image processing system. A representative immunoblot with relative signal densities is shown. Lane 1, no-treatment controls; lane 2, 600 units/ml LIF; lane 3, 50% HCCM; lane 4, 50% DFCM; .



100% 58% 51% 44%

Figure 2.5 Morphology of distal neurites treated with cholinergic switching factors

Sympathetic neurons were treated as in Fig. 2.1. Representative distal neurites photographed on day 21 in culture (2 weeks of treatment) show a central neurite cable on each of the collagen tracks (arrowheads). An extensive network of neurites surrounding the central cable is further observed in control cultures but not in cultures treated with 600 units/ml LIF (b), 50% heart cell conditioned medium (c), or 50% dermal fibroblast conditioned medium (d). Bar, 200 μm.



Chapter 3

Leukemia Inhibitory Factor and Nerve Growth Factor are Retrogradely Transported and Processed by Cultured Rat Sympathetic Neurons

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INTRODUCTION

A variety of molecules which support the survival, growth, and differentiation of neurons exert their effects by interacting with axons or axon terminals. The binding of factors to specific receptors on the neuronal membrane triggers cascades of intracellular signals which regulate not only local events at nerve terminals but also responses at the cell body which can be a long distance from the site of receptor binding. Although many second messengers and enzymatic events in signal transduction have been documented, the long-range trophic signals which link events in nerve terminals and cell bodies are unknown.

Two molecules which elicit long-range signaling in sympathetic neurons are nerve growth factor (NGF1; Paravicini et al., 1975; Levi-Montalcini, 1976; Campenot, 1977; Campenot, 1982; Korsching and Thoenen, 1983) and leukemia inhibitory factor (LIF; Ure et al., 1992). Several other events follow the binding of NGF to its receptor, including receptor-mediated endocytosis, activation of multiple protein kinases and other enzymes, and retrograde transport of NGF (reviewed by Hendry, 1992). The retrograde transport of NGF from axon terminals has been shown both in vivo (Hendry et al., 1974; Korsching and Thoenen, 1983a) and in vitro (Claude et al., 1982b). Retrograde transport has long been thought to be important for many of the NGF-mediated effects, in part due to evidence demonstrating that NGF remains largely intact after retrograde transport (Stoeckel et al., 1975; Stoeckel et al., 1976; Johnson et al., 1978; Taniuchi and Johnson, 1985). Furthermore, it has been shown that a monoclonal antibody (192-IgG) raised against the p75 neurotrophin receptor is retrogradely transported in rat sympathetic neurons and neonatal rat motor neurons, suggesting that the p75 receptor is also retrogradely transported (Taniuchi and Johnson, 1985; Yan et al., 1988). However the relevance of the transport of NGF, p75 receptor, or other molecules to retrograde signaling has not been established.

LIF is also known as cholinergic differentiation factor (CDF; Weber, 1981; Fukada, 1985). It induces the cholinergic differentiation of rat sympathetic neurons (Yamamori et al., 1989), suppresses tyrosine hydroxylase expression in trigeminal neurons (Fan and Katz, 1993), enhances the survival of chick sensory neurons (Murphy et al., 1991), and promotes the growth of rat motor neurons (Martinou et al., 1992). LIF also has multiple effects outside the nervous system (for review, see Hilton, 1992). We have previously shown that LIF is able to switch the phenotype of cultured rat sympathetic neurons from adrenergic to cholinergic when applied only to peripheral neurites (Ure et al., 1992). Therefore, LIF, like NGF, must induce a signal which travels retrogradely in neurites to reach the cell body. LIF has been shown to be transported retrogradely in sensory neurons in vivo (Hendry et al., 1992), but attempts to demonstrate retrograde transport by sympathetic neurons in vivo have been unsuccessful (Hendry et al., 1992). We now report that sympathetic neurons do retrogradely transport ^{125}I-LIF in a compartmented culture system and that the transport displays similar characteristics to ^{125}I-NGF retrograde transport. These results are consistent with a possible role of ^{125}I-LIF retrograde transport in signaling events in cell bodies.

MATERIALS AND METHODS

Culture Procedures

Superior cervical ganglia (SCG) were dissected from newborn Sprague-Dawley rat pups (supplied by University of Alberta Farm). The ganglia were chemically dissociated by separate incubations in 0.1% (w/v) trypsin (Calbiochem, La Jolla, CA) and 10 μg/ml DNAase (Sigma, St. Louis, MO), followed by mechanical dissociation as previously described (Campenot et al., 1991). General culture procedures essentially followed the methods of Hawrot and Patterson (1979). The standard culture medium was L15CO2 medium (GIBCO Laboratories, Grand Island, NY) supplemented with the prescribed additives and 6% methylcellulose.

Compartmented cultures were constructed as previously described (Campenot, 1992). Rat tail collagen was prepared and air-dried onto 35 mm tissue culture dishes (Hawrot and Patterson, 1979). Parallel collagen tracks were made by scratching the collagen substrate with a lab-made pin rake. A Teflon divider (Tyler Research Instruments, Edmonton, Alberta, Canada) was then seated on top of the tracks.

Dissociated neurons were plated into the center compartments and maintained for the first 5 days in medium containing 2.5% rat serum (prepared by Lab Animal Services, University of Alberta), 1 mg/ml ascorbic acid, 200 ng/ml 2.5S NGF (Cedarlane Laboratories Ltd., Hornby Ontario), and 10 μM cytosine arabinoside (Sigma). After 5 days, when neurites had crossed under the divider into adjacent compartments, the cell body compartments no longer received NGF. Compartments containing only neurites received medium containing 200 ng/ml NGF at all times, unless otherwise stated. Medium was changed every 3-6 days. Cultures were maintained in a 5% CO_2 atmosphere at 37°C.

Radioiodination of LIF, NGF, and Cytochrome C

Carrier-free recombinant human LIF (R&D Systems Inc., Minnesota, MN) was iodinated by the iodine monochloride method (Contreras et al., 1983; Hilton et al., 1988). First, a mixture was made consisting of 1 mCi of carrier-free $Na^{125}I$ (Amersham), 40 μl of 0.2M sodium phosphate buffer (pH 7.2), 100 μl of 5 mM ammonium bicarbonate buffer containing 1 μg of

LIF, 40% (v/v) acetonitrile, 0.125% (v/v) trifluoroacetic acid, and 0.02% (v/v) Tween 20. To start the reaction, 5 µl of 0.2 mM ICl in 2 M NaCl was added to the mixture at room temperature, while vortexing. After 1 min the reaction was stopped by the addition of 10 µl of 1 M KI. Then a 5 µl aliquot of the reaction volume was removed for TCA determination of labelling efficiencies and specific activities, and the remainder was sequentially run over Sephadex G-25M gel filtration and CM-Cellulose ion exchange columns. Specific activities of ^{125}I-LIF ranged from 800-4600 cpm/fmol. In the DA1.k cell line proliferation assay, we found that LIF-induced proliferation activity was not reduced by iodination to 4600 cpm/fmol.

NGF and Cytochrome C were iodinated by the lactoperoxidase method. The initial mixture consisted of 37 µl of 50 mM potassium phosphate buffer (pH 7.4), 3-5 µl (2.5 mCi) of Na^{125}I, and 10-20 µl (10-20 µg) of 2.5S NGF or cytochrome C. To start the reaction, 10 µl of 33 µg/ml lactoperoxidase (Sigma) and 10 µl of 0.003% H_2O_2 were then added. At the half-way point of the 1 hr, room-temperature reaction, a second identical aliquot of H_2O_2 was added. To end the reaction, 5 µl of β-mercaptoethanol (Sigma) and 320 µl of 50 mM potassium phosphate buffer containing 1 mg/ml BSA (pH 7.4) were added. TCA determination of labelling efficiencies and specific activities was performed with a 5 µl aliquot of the final reaction volume, and the remainder was run over Sephadex G-25M gel filtration columns. Specific activities of ^{125}I-NGF and ^{125}I-cytochrome C ranged from 600-1800 cpm/fmol.

Retrograde Transport and Autoradiography

Radioiodinated compounds were added to L15CO_2 medium for addition to compartmented cultures. NGF was additionally added to a final concentration of 200 ng/ml to aliquots containing ^{125}I-LIF or ^{125}I-cytochrome C, unless otherwise stated. The radiolabel-containing medium was added to side compartments containing only peripheral neurites. Prior to the addition of the ^{125}I-NGF, side compartments were first rinsed once with medium to remove residual nonlabeled NGF.

After a period allowed for retrograde transport, center compartments of cultures were analysed quantitatively and qualitatively for the transported materials. In general, medium was first removed from all compartments, followed by one rinse with cold phosphate buffered saline (PBS). In quantification studies the PBS rinse from the center compartment was pooled with the

center-compartment medium. After the PBS rinse, reducing sample buffer containing 1.3% sodium dodecyl sulfate (SDS) was added to center compartments to dissolve proximal neurites and cell bodies. After 1-3 min the extracts were collected. Medium and extract cpm were then quantified using a Wallac 1470 gamma counter.

Some samples were further characterized by electrophoresis and autoradiography. When medium components were to be analysed, only a small volume of medium (50-100 µl) was added to the compartment at the beginning of the retrograde transport experiment in order to concentrate the released radioactive material. Samples were run on gradient polyacrylamide gels, then the gels were fixed in methanol/acetic acid, dried, and exposed at -70°C to Kodak X-OMAT/AR film.

RESULTS

^{125}I-LIF Is Retrogradely, Axonally Transported By a Receptor-specific Mechanism In Sympathetic Neurons

Retrograde transport of radiolabeled factors was studied in compartmented cultures of dissociated rat sympathetic neurons. In the compartmented culture system neurites grow from cell bodies plated into a center compartment and extend into left and right side compartments. The silicone grease applied to the underside of the Teflon divider during compartment culture construction creates a sealed barrier between compartments but allows penetration of neurites. The barrier has been shown to be highly resistant to bulk fluid flow and diffusion of molecules between compartments (Campenot, 1979; Campenot and Draker, 1989; Vance et al., 1991). Quantitative comparisons following various treatments were performed on cultures within individual platings to eliminate the variability in cell and neurite density that occurs between platings. All experiments were conducted in cultures that were at least 10 days old.

Retrograde transport assays consisted of applying radiolabeled factors to peripheral neurites in side compartments continuously for a period of time, after which radioactivity in the center compartment was analysed. In some experiments, radioactivity present in the medium and cell extracts from the center compartments, made with reducing SDS-sample buffer, was separately quantified from parallel cultures at various intervals following the initial application of ^{125}I-LIF to side compartments. In a typical experiment shown in Fig. 3.1, which was confirmed by two other similar experiments, radioactivity appeared first in the cell bodies and proximal neurites and then later in the center-compartment medium bathing them. Radioactivity accumulated in the neurons until 6-9 hr, after which it appeared to reach a plateau. Radioactivity continued to accumulate in the center-compartment medium throughout the course of the experiment. In all further experiments, center compartment radiolabel was analysed following approximately 17 hr of continuous transport, at which time about 75% of the label was present in the medium.

To determine the extent to which center radiolabel accumulations represented a retrograde transport event, several control treatments were performed. In these experiments total radiolabel accumulations were compared, the total accumulations being the combined radiolabel

from cell extracts and medium. As shown in Fig. 3.2, the ^{125}I-LIF-derived radiolabel accumulation could be blocked by co-treating peripheral neurites with a 100-fold excess of unlabeled LIF. The accumulation could also be blocked by treating neurons with dinitrophenol, which uncouples oxidative phosphorylation, or with colchicine (data not shown). The low levels of accumulation accompanying these latter treatments did not exceed center radiolabel accumulation in mock cultures which contained no cells. These results, which were each confirmed in at least two other similar experiments, indicate that radiolabel accumulation in center compartments following ^{125}I-LIF application to side compartments is largely due to an energy-dependent, retrograde transport process, and suggest that an early step in the transport of ^{125}I-LIF involves the binding of ^{125}I-LIF to a saturable pool of LIF receptors.

To quantitatively compare total transport of LIF with other factors, side compartments of cultures were provided with 1 nM of either ^{125}I-LIF, ^{125}I-NGF, or ^{125}I-cytochrome C. After 17 hr, significant radiolabel accumulations were found in the center compartments following both ^{125}I-LIF and ^{125}I-NGF incubations, although to different levels (Fig. 3.3). On a molar basis, ^{125}I-NGF was transported to the highest level, reaching approximately 9 fmol per culture in the experiment shown in Fig. 3.3, while ^{125}I-LIF transport was considerably less. In four similar experiments, ^{125}I-LIF transport ranged from 5-22% of the ^{125}I-NGF transport, with the average being 14%. ^{125}I-cytochrome C was used as a control for nonspecific transport since it does not bind to specific cell surface receptors. Center compartment accumulation of ^{125}I-cytochrome C-derived radiolabel did not exceed accumulations of ^{125}I-cytochrome C or ^{125}I-LIF in mock cultures which lacked cells. Therefore, retrograde transport following non-specific uptake (e.g. pinocytosis) cannot explain the observed accumulations of ^{125}I-LIF and ^{125}I-NGF.

The Rate of ^{125}I-LIF Transport Is Unaffected By NGF Concentration

NGF supports the survival and growth of the neurons, while LIF alters their neurotransmitter phenotype. Although these effects are very different, both molecules are internalized by receptor-mediated endocytosis and presumably share many of the same mechanisms. Therefore, the uptake and transport of one of the molecules might be expected to change that of the other molecule. To examine whether the magnitude of ^{125}I-LIF transport was related to the NGF concentration in the medium bathing the peripheral neurites, 1 nM ^{125}I-LIF

was applied in combination with either 10 or 200 ng/ml NGF to peripheral neurites in parallel cultures. For four days prior to the experiment, neurites in side compartments had been maintained in 10 ng/ml NGF rather than the normal 200 ng/ml to reduce the possible interference of residual NGF (Hawrot, 1982). Following incubation of peripheral neurites for 20.5 hr, no difference in receptor-specific, ^{125}I-LIF retrograde transport was observed between the two treatments (Fig. 3.4). These cultures had no NGF supplied in the center-compartment medium. In other cultures, inclusion of 200 ng/ml NGF in the center compartment during the transport period also had no affect upon ^{125}I-LIF transport. These observations were confirmed in one additional experiment. In examining the reverse situation, whether LIF affects NGF transport, preliminary experiments suggest that ^{125}I-NGF transport does not change in the presence of LIF.

^{125}I-NGF and ^{125}I-LIF transport Is Reduced Following Neuronal Pretreatment With LIF

The presence of LIF did not appear to affect ^{125}I-NGF retrograde transport (see above). However, it is possible that long-term, LIF pretreatment would affect the subsequent transport of ^{125}I-NGF or ^{125}I-LIF, since the phenotypic change in cultured sympathetic neurons from adrenergic to cholinergic develops over many days (Ure et al., 1992). We tested this possibility by pretreating cultures with LIF for 6 days prior to performing ^{125}I-NGF and ^{125}I-LIF retrograde transport assays. Six days was chosen as the LIF pretreatment period as this is a time at which the switch to a cholinergic phenotype is reliably detected (Ure et al., 1992). As shown in Fig. 3.5, after pretreating the neurons with 600 units/ml LIF, and after incubating peripheral neurites with either ^{125}I-NGF or ^{125}I-LIF, center radiolabel accumulations were significantly reduced, compared to the same assay in cultures that were not pretreated. A reduction of similar magnitude was also observed after pretreatment with 5 ng/ml of purified LIF (data not shown). The reduction in ^{125}I-LIF transport occurred whether pretreatment occurred in the side compartments or in the center compartment. Since LIF receptor sequestration in peripheral neurites would not have occurred after center-compartment application of LIF, this event could not account for the reduced ^{125}I-LIF transport. Rather, it appears that the reduction in transport of both ^{125}I-NGF or ^{125}I-LIF is the result of a general phenotypic change in the neurons.

^{125}I-LIF and ^{125}I-NGF Undergo Intracellular Proteolysis and Release

To investigate the nature of the radiolabel associated with the neurons and medium during or following retrograde transport of ^{125}I-LIF and ^{125}I-NGF, samples of cell extract and medium were analyzed by SDS-PAGE and autoradiography. Reducing sample buffer was used to make the cell extracts as well as to prepare medium for SDS-PAGE. After 15-18 hr of transport, both ^{125}I-LIF and ^{125}I-NGF in center-compartment extracts were present as apparently intact and partially processed forms (Fig. 3.6 and 3.7). Two ^{125}I-LIF species were observed in extracts, one migrating at the same position as intact ^{125}I-LIF and the second, in approximately equal proportion, migrating at a slightly lower molecular weight. The major form of ^{125}I-NGF in extracts migrated at the same molecular weight as intact ^{125}I-NGF, although 3 faster-migrating forms were also present. As ^{125}I-NGF decreased in size the radiolabel intensities also decreased.

To control for the unlikely possibility that the lower molecular weight species in the cell extract were produced artifactually during the harvest procedure, intact ^{125}I-LIF or ^{125}I-NGF were dissolved in sample buffer and applied to the center compartments of cultures not previously used in a retrograde transport assay. No degradation of the factors was observed under these conditions, suggesting that the processing observed after retrograde transport had occurred within intact cells (Fig. 3.6).

When radiolabel released into the medium was analyzed by SDS-PAGE and autoradiography, the only observed species migrated at the phenol red dye front on high concentration gradient gels. Dye-front signals have been shown to largely represent single radiolabeled tyrosine residues (Layer and Shooter, 1983; Eveleth and Bradshaw, 1992). The radiolabel in the medium was not precipitable with trichloroacetic acid, which is consistent with extensive proteolysis of the factors (data not shown), and which is also consistent with observations using conditioned medium from PC12 cells (Layer and Shooter, 1983). To test the possibility that ^{125}I-NGF was degraded by cellular enzymes released into the medium, non-radioactive medium from center-compartments was incubated with intact ^{125}I-NGF for periods up to one hour. Under these conditions no degradation of ^{125}I-NGF was observed (data not shown). Taken together, these results show that retrograde transport of ^{125}I-LIF and ^{125}I-NGF is accompanied by the cellular accumulation of intermediate, proteolysed products of these factors.

These data further suggest that extensive degradation and release of at least some, and perhaps all of the products into the medium completes the cellular processing of ^{125}I-LIF and ^{125}I-NGF.

DISCUSSION

It was previously shown that LIF can induce the switch from an adrenergic to cholinergic phenotype in rat sympathetic neurons when applied only to neurites in side compartments (Ure et al., 1992). In the present study we have found that retrograde transport of LIF occurs when the factor is applied to peripheral neurites. The process is receptor-mediated and is accompanied by processing of the ligand and accumulation of cell-associated, processed species. We have similarly observed ligand processing and accumulation of ^{125}I-NGF following retrograde transport.

Retrograde transport of LIF has previously been investigated in vivo (Hendry et al., 1992). Although LIF was reported to be transported in sensory neurons, retrograde transport by sympathetic neurons was not detected (Hendry et al., 1992). An important consideration is that the above in vivo study focused on mature, adult neurons, while our studies are on actively-growing, neonatally-derived neurons. The observed lack of transport in vivo could also be related, in part, to our observation of low transport capacity, approximately 6-fold less than NGF. The compartmented culture system is especially well-suited for detecting and quantifying retrograde transport in that, unlike in vivo experimentation, labeled factors can be supplied to peripheral axons for sustained periods, and all of the label released by the cell bodies can be collected. A report of increased retrograde transport of ciliary neurotrophic factor (CNTF) following peripheral nerve injury (Curtis et al., 1993) raises the possibility that the LIF transport which we observe may be enhanced by the axotomy that occurs when the neurons are removed from the animal.

The time course of radiolabel accumulations in cell extracts and medium and the autoradiographic identification of the radiolabeled species is consistent with a model in which neurons retrogradely transport LIF, store it for a period in both intact and partially proteolysed forms, and then completely degrade and release it into the extracellular environment. Our data on NGF processing and transport as well as that by Claude et al. (1982b) suggest a similar model for NGF. It is difficult to interpret the meaning of the various label intensities of the cell extract species, since their accumulation in the intracellular compartments and, in turn, our ability to detect them is related to variables such as residence times in the intracellular compartments, the number of molecules passing through them, and the number of iodinated residues per molecule.

Nevertheless, the presence of the very low molecular weight species only in the medium and not in the cell extract suggests that the exit rate of ligand components from the final site of degradation must be relatively rapid.

The cellular accumulation of intermediate products of NGF and LIF processing following retrograde transport has not been previously observed in any system. In studies where the products of ^{125}I-NGF retrograde transport have been studied by SDS-PAGE, low molecular weight forms in extracts of superior cervical ganglia have not been reported (Stoeckel et al., 1976; Johnson et al., 1978; Taniuchi and Johnson, 1985). However, in two of the reports the data do not exclude the possibility that degradation products did accumulate, albeit in very small quantities (Stoeckel et al., 1976; Johnson et al., 1978). In PC12 cells, intermediate products of ^{125}I-NGF degradation have been reported not to accumulate, although small amounts of terminally degraded ^{125}I-NGF were visualized in cell extracts (Eveleth and Bradshaw, 1992). As with the LIF transport, conflicting observations may be related to the differences between in vivo and culture models, and/or to the high sensitivity of our culture system.

Intermediate, intracellular products of ligand processing have been observed in the case of internalization of epidermal growth factor (EGF) by fibroblasts (Matrisian et al., 1984; Planck et al., 1984; Renfrew and Hubbard, 1991), insulin by hepatocytes (Assoian and Tager, 1982; Pease et al., 1985; Hamel et al., 1988; Williams et al., 1990), and β-subunit of human choriogonadotropin (hCG) in Leydig tumor cells (Ascoli, 1982). The role of limited proteolysis is not known but there is evidence that it may act as an off-switch to signaling by the receptor by inducing ligand-receptor dissociation (Carpenter et al., 1975; Ascoli, 1982; Planck et al., 1984; Wiley et al., 1985; Hamel et al., 1988; Backer et al., 1990). By analogy, processing of LIF and NGF that we have observed may reduce or terminate the signals generated when these molecules bind to their receptors.

Ultrastructural examination of the location of intracellular NGF following retrograde transport both in vivo and in vitro reveal its presence in several organelle types in cell bodies, including lysosomes, multivesicular bodies (MVB), smooth vesicles, and smooth endoplasmic reticulum (Schwab and Thoenen, 1977; Claude et al., 1982a; Claude et al., 1982b). In cultured rat hippocampal neurons, fluid-phase markers have also been observed to be associated with several organelle types during and following transport (Parton et al., 1992). Given the variety of organelles in which NGF has been previously observed, and the variety of processed forms which

we have observed, it is tempting to speculate that multiple processing pathways might exist for NGF and possibly for LIF. Endosomes have traditionally been thought to act principally as a transport reservoir, but there is considerable evidence also implicating them in the partial proteolysis of several internalized molecules (Matrisian et al., 1984; Diment and Stahl, 1985; Pease et al., 1985; Schaudies et al., 1987; Hamel et al., 1988; Diment et al., 1989; Backer et al., 1990; Doherty et al., 1990; Blum et al., 1991; Renfrew and Hubbard, 1991). In some cases where endosomes have been shown to have a proteolytic function, different degradation pathways for the same molecule have been reported to exist within a single cell type (Misbin et al., 1983; Williams et al., 1990; Sorkin et al., 1991).

NGF is discretely localized in the adult rat, and its abundance in target fields of sympathetic neurons correlates with the extent of innervation (Korsching and Thoenen, 1983b; Korsching and Thoenen, 1988). Messenger RNA for LIF is also discretely localized in the adult rat, including its presence in footpads which are targets of sympathetic neurons. It is worthwhile to know whether either of the factors exert an effect on signaling by the other factor, especially since there is very little overlap in the types of effects the factors have on sympathetic neurons. Our results revealing no difference in ^{125}I-LIF transport over a 20-fold range in NGF concentration suggests that LIF-induced retrograde signaling is unaffected by accessibility to NGF, assuming that the signaling is directly correlated with LIF retrograde transport. This postulate may not apply at very low NGF concentrations when neuronal viability may be compromised. In contrast to the above results, retrograde signaling by NGF might be reduced under conditions in which sympathetic neurons are chronically exposed to LIF or related factors, such as in the adult rat footpad (reviewed by Rao and Landis, 1993). We found that ^{125}I-NGF transport was significantly reduced in neurons that had been pretreated for 6 days with LIF. However, it is not yet known whether NGF-dependent, retrograde signaling events such as gene transcription or survival are affected by chronic LIF exposure.

Our observations of LIF retrograde transport support the hypothesis that ligand transport to cell bodies is important for induction of cell body events. It will be interesting to discover whether some factors exert retrograde effects without themselves being transported. The possibility still exists that retrograde transport is an obligatory event following receptor binding whose function is only to catabolize ligand that has already induced local signals. In that case,

long-range signals must be generated by other mechanisms, perhaps by the transport of other second messengers or of receptor whose activation is sustained even after ligand dissociation.

REFERENCES

Ascoli, M. (1982). Internalization and degradation of receptor-bound human choriogonadotropin in Leydig tumor cells. J. Biol. Chem. 257, 13306-13311.

Assoian, R. K., and Tager, H. S. (1982). Peptide intermediates in the cellular metabolism of insulin. J. Biol. Chem. 257, 9078-9085.

Backer, J. M., Kahn, C. R., and White, M. F. (1990). The dissociation and degradation of internalized insulin occur in the endosomes of rat hepatoma cells. J. Biol. Chem. 265, 14828-14835.

Blum, J. S., Fiani, M. L., and Stahl, P. D. (1991). Proteolytic cleavage of ricin A chain in endosomal vesicles. J. Biol. Chem. 266, 22091-22095.

Campenot, R. B. (1977). Local control of neurite development by nerve growth factor. Proc. Natl. Acad. Sci. USA 74, 4516-4519.

Campenot, R. B. (1979). Independent control of the local environment of somas and neurites. Methods Enzymol. 28, 302-307.

Campenot, R. B. (1982). Development of sympathetic neurons in compartmentalized cultures. I. Local control of neurite growth by nerve growth factor. Dev. Biol. 93, 1-12.

Campenot, R. B., and Draker, D. D. (1991). Growth of sympathetic nerve fibers in culture does not require extracellular calcium. Neuron 3, 733-743.

Campenot, R. B., Walji, A. H., and Draker, D. D. (1991). Effects of sphingosine, staurosporine, and phorbol ester on neurites of rat sympathetic neurons growing in compartmented cultures. J. Neurosci. 11, 1126-1139.

Campenot, R. B. (1992). Compartmented culture analysis of nerve growth. In Cell-Cell Interactions: A Practical Approach. Stevenson, B., Paul, D., and Gallin, W. eds. Oxford U. Press. pp. 275-298.

Carpenter, G., Lembach, K. J., Morrison, M. M., and Cohen, S. (1975). Characterization of the binding of 125I-labeled epidermal growth factor to human fibroblasts. J. Biol. Chem. 250, 4297-4304.

Claude, P., Hawrot, E., and Parada, I. (1982a). Ultrastructural studies on the intracellular fate of 125I-nerve growth factor in cultured rat sympathetic neurons. J. Cell. Biochem. 20, 1-13.

Claude, P., Hawrot, E., Dunis, D. A., and Campenot, R. B. (1982b). Binding, internalization, and retrograde transport of 125I-nerve growth factor in cultured rat sympathetic neurons. J. Neurosci. 2, 431-442.

Contreras, M. A., Bale, N. F., and Spar, I. L. (1983). Iodine monochloride (ICl) iodination techniques. Meth. Enzymol. 92, 277-292.

Curtis, R., Adryan, K. M., Zhu, Y., Harkness, P. J., Lindsay, R. M., and DiStefano, P. S. (1993). Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature 365, 253-255.

Diment, S., and Stahl, P. (1985). Macrophage endosomes contain proteases which degrade endocytosed protein ligands. J. Biol. Chem. 260, 15311-15317.

Diment, S., Martin, K. J., and Stahl, P. D. (1989). Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. J. Biol. Chem. 264, 13403-13406.

Doherty, J. J., Kay, D. G., Lai, W. H., Posner, B. I., and Bergeron, J. J. M. (1990). Selective degradation of insulin within rat liver endosomes. J. Cell Biol. 110, 35-42.

Eveleth, D. D., and Bradshaw, R. A. (1992). Nerve growth factor nonresponsive pheochromocytoma cells: Altered internalization results in signaling dysfunction. J. Cell Biol. 117, 291-299.

Fan, G., and Katz, D. M. (1993). Non-neuronal cells inhibit catecholaminergic differentiation of primary sensory neurons: role of leukemia inhibitory factor. Development 118, 83-93.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc. Natl. Acad. Sci. USA 82, 8795-8799.

Hamel, F. G., Posner, B. I., Bergeron, J. J. M., Frank, B. H., and Duckworth, W. C. (1988). Isolation of insulin degradation products from endosomes derived from intact rat liver. J. Biol Chem. 263, 6703-6708.

Hawrot, E. (1982). Characteristics of the association of nerve growth factor with primary cultures of rat sympathetic neurons. J Neurosci. Res. 8, 213-224.

Hawrot, E., and Patterson, P. H. (1979). Long-term cultures of dissociated sympathetic neurons. Meth. Enzymol. 28, 574-584.

Hendry, I. A. (1992). Retrograde factors in peripheral nerves. Pharmac. Ther. 56, 265-285.

Hendry, I. A., Stockel, K., Thoenen, H., and Iversen, L. L. (1974). The retrograde transport of nerve growth factor. Br. Res. 68, 103-121.

Hendry, I. A., Murphy, M., Hilton, D. J., Nicola, N. A., Bartlett, P. F. (1992). Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. J. Neurosci. 12, 3427-3434.

Hilton, D. J. (1992). LIF:Lots of interesting functions. TIBS 17, 72-76.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988). Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. Proc. Natl. Acad. Sci. USA 85, 5971-5975.

Johnson, E. M. Jr., Andres, R. Y., and Bradshaw, R. A. (1978). Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I]NGF. Br. Res. 150, 319-331.

Korsching, S., and Thoenen, H. (1983a). Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. Neurosci. Lett. 39, 1-4.

Korsching, S., and Thoenen, H. (1983b). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of sympathetic innervation. Proc. Natl. Acad. Sci. USA 80, 3513-3516.

Korsching, S., and Thoenen, H. (1988). Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev. Biol. 126, 40-46.

Layer, P. G., and Shooter, E. M. (1983). Binding and degradation of nerve growth factor by PC12 pheochromocytoma cells. J. Biol. Chem. 258, 3012-3018.

Levi-Montalcini, R. (1976). The nerve growth factor: Its role in growth, differentiation, and function of the sympathetic adrenergic neuron. Prog. Brain Res. 45, 235-258.

Martinou, J. C., Martinou, I., and Kato, A. C. (1992). Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. Neuron 8, 737-744.

Matrisian, L. M., Planck, S. R., and Magun, B. E. (1984). Intracellular processing of epidermal growtnh factor. J. Biol. Chem. 259, 3047-3052.

Misbin, R. I., Almira, E. C., and Buynitzky, S. J. (1983). Insulin metabolism in rat hepatocytes. J. Biol. Chem. 258, 2157-2162.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. Proc. Natl. Acad. Sci. USA 88, 3498-3501.

Paravicini, U., Stoeckel, K., and Thoenen, H. (1975). Biological importance of retrograde axonal transport of nerve growth factor in adrenergic neurons. Br. Res. 84, 279-291.

Parton, R. G., Simons, K., and Dotti, C. G. (1992). Axonal and dendritic endocytic pathways in cultured neurons. J. Cell Biol. 119, 123-137.

Pease, R. J., Smith, G. D., and Peters, T. J. (1985). Degradation of endocytosed insulin in rat liver is mediated by low-density vesicles. Biochem. J. 228, 137-146.

Planck, S. R., Finch, J. S., and Magun, B. E. (1984). Intracellular processing of epidermal growth factor. J. Biol. Chem. 259, 3053-3057.

Rao, M. S., and Landis, S. C. (1993). Cell interactions that determine sympathetic neuron transmitter phenotype and the neurokines that mediate them. J. Neurobiol. 24, 215-232.

Renfrew, C. A., and Hubbard, A. L. (1991). Sequential processing of epidermal growth factor in early and late endosomes of rat liver. J. Biol. Chem. 266, 4348-4356.

Schaudies, R. P., Gorman, R. M., Savage, C. R., and Poretz, R. D. (1987). Proteolytic processing of epidermal growth factor within endosomes. Bioch. Biophys. Res. Comm. 143, 710-715.

Schwab, M., and Thoenen, H. (1977). Selective trans-synaptic migration of tetanus toxin after retrograde axonal transport in peripheral sympathetic nerves: A comparison with nerve growth factor. Br. Res. 122, 459-474.

Sorkin, A., Krolenko, S., Kudrjavtceva, N., Lazebnik, J., Teslenko, L., Soderquist, A. M., and Nikolsky, N. (1991). Recycling of epidermal growth factor-receptor complexes in A431 cells: Identification of dual pathways. J. Cell Biol. 112, 55-63.

Stoeckel, K., Schwab, M., and Thoenen, H. (1975). Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: A biochemical and morphological study. Br. Res. 89, 1-14.

Stoeckel, K., Guroff, G., Schwab, M., and Thoenen, H. (1976). The significance of retrograde axonal transport for the accumulation of systemically administered nerve growth factor (NGF) in the rat superior cervical ganglion. Br. Res. 109, 271-284.

Taniuchi, M., and Johnson, E. M. (1985). Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directed against the rat nerve growth factor receptor. J. Cell Biol. 101, 1100-1106.

Ure, D. R., Campenot, R. B., and Acheson, A. (1992). Cholinergic differentiation of rat sympathetic neurons in culture: Effects of factors applied to distal neurites. Dev. Biol. 154, 388-395.

Vance, J. E., Pan, D., Vance, D. E., and Campenot, R. B. (1991). Biosynthesis of membrane lipids in rat axons. J. Cell Biol. 115, 1061-1068.

Weber, M. J. (1981). A diffusible factor responsible for the determination of cholinergic functions in cultured sympathetic neurons. J. Biol Chem. 256, 3447-3453.

Wiley, H. S., VanNostrand, W., McKinley, D. N., and Cunningham, D. D. (1985). Intracellular processing of epidermal growth factor and its effect on ligand-receptor interactions. J. Biol. Chem. 260, 5290-5295.

Williams, F. G., Johnson, D. E., and Bauer, G. E. (1990). [125I]-insulin metabolism by the rat liver in vivo: Evidence that a neutral thiol-protease mediates rapid intracellular insulin degradation. Metabolism 39, 231-241.

Yamamori, T. (1991). Localization of cholinergic differentiation factor/leukemia inhibitory factor mRNA in the rat brain and peripheral tissues. Proc. Natl. Acad. Sci. USA 88, 7298-7302.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M. J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science 246, 1412-1416.

Yan, Q., Snider, W. D., Pinzone, J. J., and Johnson, E. M. Jr. (1988). Retrograde transport of nerve growth factor (NGF) in motoneurons of developing rats: Assessment of potential neurotrophic effects. Neuron 1, 335-343.

FIGURES

Figure 3.1 Time course of radiolabel accumulation in center-compartment media and extracts following ^{125}I-LIF retrograde transport

0.25 nM ^{125}I-LIF was applied to side compartments of 14 day-old cultures. At the indicated times, center-compartment media and cell extracts were collected and assayed separately for radiolabel content (amol). Individual points represent means (± SEM) of 3 cultures, except at 12 hr where the mean of 2 cultures is provided. Except for 12 hr, errors fall within the symbols when error bars are absent.

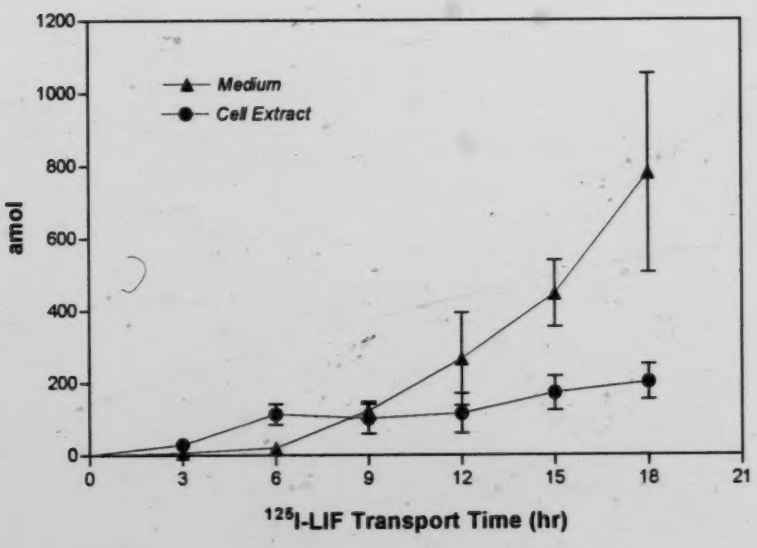

1200
1000
800
600
400
200
0
amol
Medium
Cell Extract
0
3
6
9
12
15
18
21
125I-LIF Transport Time (hr)

Figure 3.2 Characteristics of ^{125}I-LIF retrograde transport

1 nM ^{125}I-LIF was applied to side compartments alone or in combination with 100 nM unlabeled LIF in side compartments, or with 5 mM dinitrophenol in all compartments. These treatments were applied to 28 day-old neuronal cultures. Mock cultures (no cells plated) were given ^{125}I-LIF for comparison. After 17 hr, center-compartment media and cell extracts were harvested, and the combined radiolabel contents quantified and converted to amol/center compartment. Bars represent means (± SEM) of 3 cultures.

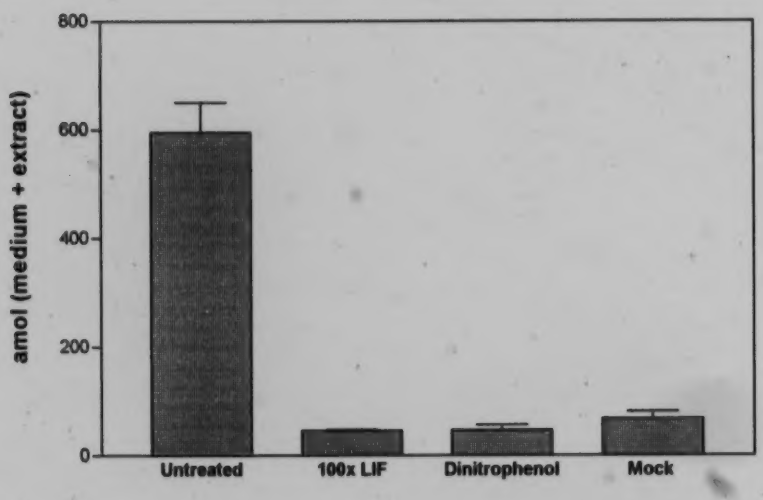

amol (medium + extract)
800
600
400
200
0
Untreated
100x LIF
Dinitrophenol
Mock

Figure 3.3 Center-compartment accumulations of radiolabelled factors

Radiolabeled factors were applied at a concentration of 1 nM to side compartments of neuronal cultures (35 days-old) or mock cultures (no cells plated). After 17 hr, center-compartment media and cell extracts were harvested and the combined radiolabel contents quantified and converted to amol/center compartment. Bars represent means (± SEM) of 3 or 4 cultures, except for cytochrome C treatments (2 cultures each).

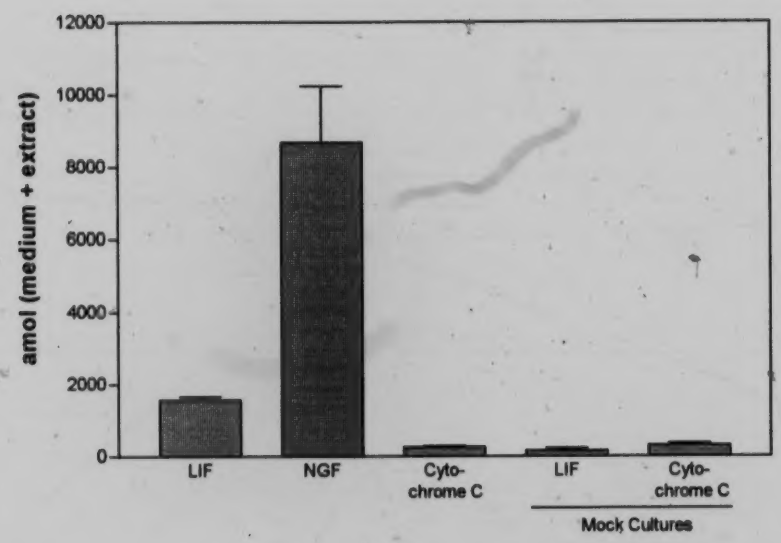

amol (medium + extract)
12000
10000
8000
6000
4000
2000
0
LIF
NGF
Cyto-chrome C
LIF
Cyto-chrome C
Mock Cultures

Figure 3.4 Effect of NGF concentration on ^{125}I-LIF transport rate

For 4 days prior to the retrograde transport assay, peripheral neurites were incubated with 10 ng/ml NGF. For the transport assay, 1 nM ^{125}I-LIF was then applied to side compartments (20 day-old cultures) in combination with either 10 or 200 ng/ml NGF. In one experimental group, 200 ng/ml NGF was also added to center compartments. In other cultures, 100 nM LIF was additionally added to side compartments. After 20.5 hr, center-compartment media and cell extracts were harvested, and the combined radiolabel contents quantified and converted to amol/center compartment. Bars represent means (± SEM) of 4 cultures, except for cultures which received excess LIF (2 cultures each).

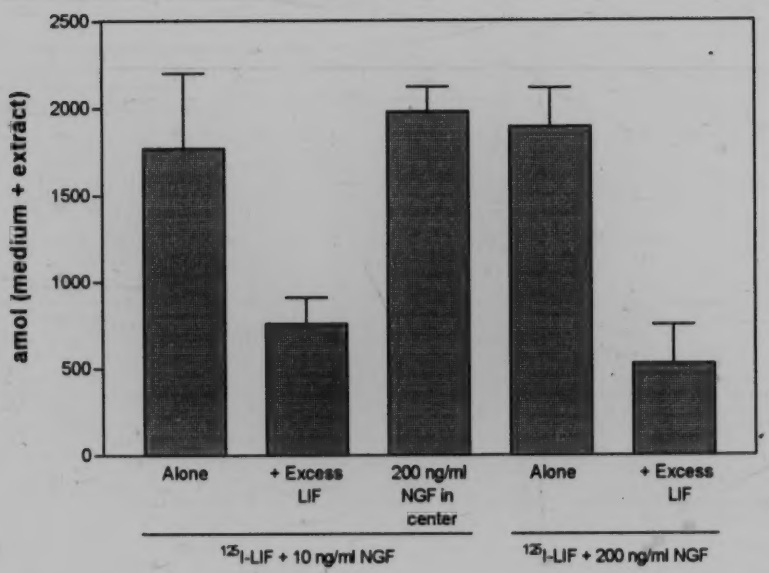

2500
2000
1500
1000
500
0
amol (medium + extract)
Alone
+ Excess LIF
200 ng/ml NGF in center
Alone
+ Excess LIF
125I-LIF + 10 ng/ml NGF
125I-LIF + 200 ng/ml NGF

Figure 3.5 Effect of LIF pretreatment on ^{125}I-NGF and ^{125}I-LIF transport

Cultures were untreated or pretreated in the indicated compartments with 600 units/ml LIF (DA1.k proliferation assay) for 6 days prior to the retrograde transport assays. For the transport assays, 7.7 nM ^{125}I-NGF was applied to side compartments of 15 day-old cultures for 18 hr, or 0.25 nM ^{125}I-LIF was applied to side compartments of 11 day-old cultures for 15 hr. Center-compartment media and cell extracts were harvested, and the combined radiolabel contents quantified and converted to amol/center compartment. Solid bars: ^{125}I-NGF transport; open bars: ^{125}I-LIF transport. Bars represent means (± SEM) of 4-5 cultures.

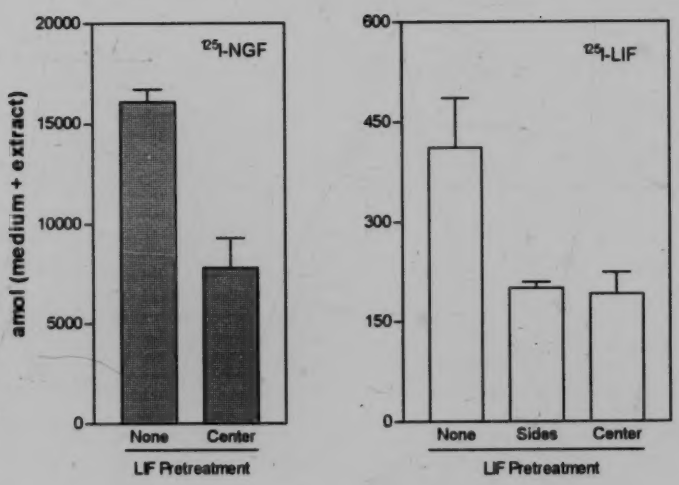

amol (medium + extract)
20000
15000
10000
5000
0
125I-NGF
None
Center
LIF Pretreatment
600
450
300
150
0
125I-LIF
None
Sides
Center
LIF Pretreatment

Figure 3.6 Electrophoretic migration and autoradiography of retrogradely transported ^{125}I-LIF

A. After 17 hr of ^{125}I-LIF retrograde transport, cell extracts (made with reducing sample buffer) and medium from the center compartments of two, 13 day-old cultures were combined and electrophoresed through a 10% linear polyacrylamide gel. Lane 1, radioactive standards; lane 2, intact ^{125}I-LIF from side compartments following the transport assay; lane 3, intact ^{125}I-LIF incubated for 1 hr with reducing sample buffer and cell bodies/proximal neurites from a culture not previously exposed to ^{125}I-LIF; lanes 5 and 6, center-compartment samples following ^{125}I-LIF transport. After fixation and drying, the gel was exposed to autoradiographic film for 17 days. B. A shorter exposure of lanes 2 and 3 from Panel A, showing that only the single intact form of ^{125}I-LIF is present.

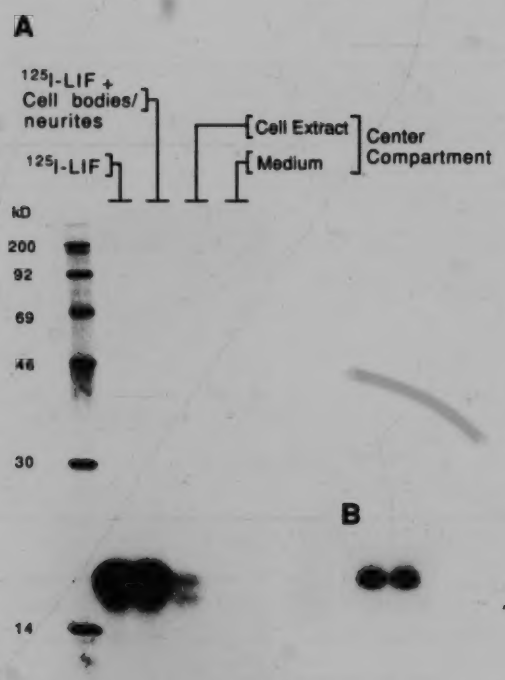
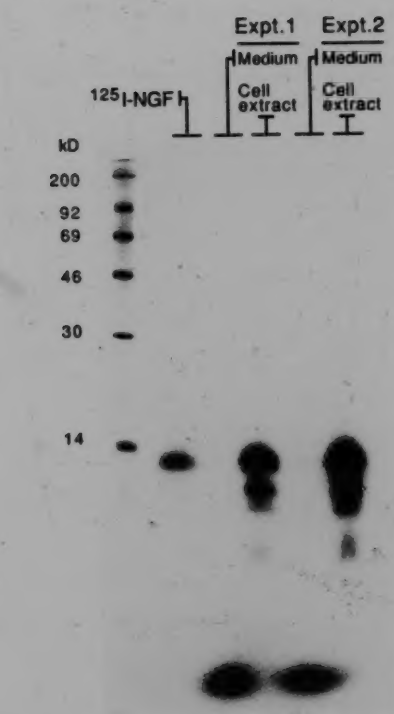

A
^{125}I-LIF +
Cell bodies/
neurites
^{125}I-LIF
Cell Extract
Medium
Center
Compartment
kD
200
92
69
46
30
14
B

Figure 3.7 Electrophoretic migration of retrogradely transported ^{125}I-NGF

After 17 hr of retrograde transport, cell extracts and aliquots of medium from two, 18 day-old cultures were electrophoresed through a 13-22% gradient polyacrylamide gel. Intact ^{125}I-NGF was additionally loaded. Experiment 2 is essentially identical to Expt. 1 except that the culture was pretreated for 6 days with LIF, a treatment found not to alter ^{125}I-NGF degradation. The gel was fixed, dried, and exposed to autoradiographic film for 21 days.


Expt.1
Expt.2
Medium
Medium
125I-NGF
Cell extract
Cell extract
kD
200
92
69
46
30
14

Chapter 4

Retrograde Transport and Steady-State Distribution of ^{125}I-NGF in Rat Sympathetic Neurons in Compartmented Cultures

[Accepted for publication in 1996 in Journal of Neuroscience, in press]

INTRODUCTION

Neurotrophic factors are internalized by axons and retrogradely transported to cell bodies. This was first demonstrated by the finding that ^{125}I-NGF injected into the eye is delivered to neuronal cell bodies of the sympathetic superior cervical ganglion (SCG) by axons innervating the iris (Hendry et al., 1974a,b; Stöckel et al., 1974; Stöckel and Thoenen, 1975; Johnson et al., 1978). It was later confirmed that endogenous NGF produced in targets of sympathetic axons is retrogradely transported and accumulates to high levels in sympathetic ganglia (Korsching and Thoenen, 1983a,b, 1988; Palmatier et al., 1984; Nagata et al., 1987). NGF is mostly intact following its transport (Hendry et al., 1974a; Stöckel et al., 1974, 1976; Johnson et al., 1978; Dumas et al., 1979; Ure et al., 1994). These observations have been interpreted as suggesting that NGF directly participates in signaling in cell bodies, likely as part of a transported ligand-receptor complex. This vesicular transport hypothesis of NGF signaling is supported by correlations between NGF retrograde transport and changes in enzyme activity and gene expression which occur in cell bodies (Paravicini et al., 1975; Stoeckel and Thoenen, 1975; Kessler and Black, 1979; Goedert et al., 1981; Miller et al., 1994).

The vesicular transport hypothesis of NGF signaling has been interpreted as implying that NGF should be concentrated in the cell bodies of responsive neurons. In calculations of transport velocity an assumption is made that NGF enters the transport pathway rapidly after binding to receptors on axon terminals and that transport rates are limited only by the velocity of the transport mechanism (Hendry et al., 1974a,b; Johnson et al., 1978). It is also assumed that NGF is neither degraded nor released intact while en route to cell bodies. However, some NGF is contained in lysosomal organelles in axons (Claude et al., 1982a; Bernd and Greene, 1983), and the possibility that it may be degraded or released before reaching cell bodies has not been tested.

Rigorous tests of these assumptions have been difficult to perform in retrograde transport models in vivo. In the present study we have examined these assumptions using compartmented

cultures of sympathetic neurons given ^{125}I-NGF on their distal axons, which allowed determination of: the amount of NGF associated with distal axons, the amount transported per hour to cell bodies, the amount accumulated by cell bodies at steady-state, and where NGF or its degradation products are released.

MATERIALS AND METHODS

Materials

Newborn Sprague-Dawley rats were supplied by University of Alberta Farm. Trypsin was obtained from Calbiochem (La Jolla, CA). L15CO_2 culture medium was obtained from GIBCO Laboratories (Grand Island, NY). Adult rat serum was prepared from whole blood supplied by Lab Animal Services, University of Alberta. Rat tail collagen was prepared by the method of Hawrot and Patterson (1979). 2.5S NGF was purchased from Cedarlane Laboratories Ltd. (Hornby, Ontario). Teflon dividers were purchased from Tyler Research Instruments (Edmonton, Alberta). $Na^{125}I$ was purchased from Amersham (Oakville, Ontario). PD-10 sephadex G-25M columns were purchased from Pharmacia Biotech (Baie d'Urfé, Quebec). Reagents whose suppliers are not stated were obtained from Sigma (St. Louis, MO).

Compartmented Cultures

Sympathetic neurons were isolated from the superior cervical ganglia of newborn rats by 0.1% (w/v) trypsin incubation and mechanical dissociation, as previously described (Campenot et al., 1991). The standard culture medium was L15CO_2 supplemented with the prescribed additives and 6% methylcellulose. Compartmented cultures were constructed as previously described (Campenot, 1992). Briefly, rat tail collagen was air-dried onto 35 mm culture dishes and then parallel scratches made in the substrate. Teflon dividers of either 3-compartment or 5-compartment design were seated onto the collagen tracks using silicon vacuum grease. Cell suspension was plated into a single compartment of each culture (2000-3000 neurons/culture), and axons extended into adjacent compartments containing NGF. For the first 6 days following plating, medium additives in the cell body compartment/proximal axon compartment included 2.5% rat serum, 1 mg/ml ascorbic acid, 10-200 ng/ml NGF, and 10 μM cytosine arabinoside. Thereafter cell body compartments received only rat serum and ascorbic acid. Axon compartments received only NGF as a medium additive at concentrations of 10-200 ng/ml throughout the entire culture period. Medium was changed every 4-6 days. Cultures were maintained in a 5% CO_2 atmosphere at 37°C.

Radioiodination of NGF

NGF was radioiodinated by the lactoperoxidase method. The following ingredients were mixed at room temperature for 1 hr: 3-5 µl (1.5 mCi) $Na^{125}I$, 10 µl (10 µg) NGF, 37 µl 0.5M potassium phosphate buffer (pH 7.4), 10 µl 33 µg/ml lactoperoxidase, and 10 µl 0.003% H_2O_2. After the first 30 min of incubation, an additional 10µl of H_2O_2 was added. The reaction was terminated with 5 µl ß-mercaptoethanol and 415 µl 1 mg/ml BSA in potassium phosphate buffer. Specific activities and labeling efficiencies were calculated after acid precipitation (20% trichloroacetic) of an aliquot of the reaction mixture. Free iodine was separated from ^{125}I-NGF using Sephadex G-25M gel filtration columns. Radioactivity was measured using a Wallac 1470 gamma counter. Specific activities averaged 151 cpm/pg and ^{125}I-NGF was used within 3 weeks of iodination. Molar concentrations of NGF were based on a molecular weight of 26,000.

Retrograde Transport Assay and Sample Analysis

Three different designs of compartmented cultures were used, based on 2 models of Teflon dividers. The cultures differed principally by the number of compartments through which axons could unidirectionally extend (Fig. 1). All retrograde transport assays were performed by applying ^{125}I-NGF to distal axons in compartments most distal to the cell body compartment and incubating at 37°C to allow time for the neurons to retrogradely transport ^{125}I-NGF. Radioactivity from cell body/proximal axon compartments (transported ^{125}I-NGF) and in some experiments from the intermediate axon compartments (5-compartment cultures) was quantified following the incubation. Medium was collected and combined with a cold PBS rinse, then cell extracts were made using reducing sample buffer containing 1.3% sodium dodecyl sulfate. Total transported ^{125}I-NGF was the combined radioactivity from cell bodies/proximal axons plus the medium bathing them. Background diffusion of ^{125}I-NGF under the Teflon divider was very low. For example, when ^{125}I-NGF was applied at a concentration of 1500 cpm/µl (10 ng/ml) to both side compartments of 3-compartment dishes lacking cells, typically radioactivity accumulated in the center compartment at a rate of 10 cpm/hr (0.06 pg/hr). After a 24 hr incubation in these mock cultures the final ^{125}I-NGF concentration in center compartments resulting from background diffusion was typically about 1 pM. To compete the uptake of ^{125}I-NGF directly by

the cell bodies, 8 nM NGF was included in the medium bathing cell bodies/proximal axons in all experiments. The presence of 8 nM NGF in this compartment was found not to affect the retrograde transport rate. For example, in one experiment 10.4% of the axonal ^{125}I-NGF was transported per hour (transport rate) when 8 nM NGF was included in the cell body compartment, whereas the transport rate was 9.8%/hr when NGF was absent from this compartment. The fraction of cellular ^{125}I-NGF found in cell bodies/proximal axons also was unaffected by including NGF in the cell body compartment. Results from a second, similar experiment were consistent with these conclusions.

The amount of ^{125}I-NGF associated with, or dissociated from distal axons was determined following the overnight ^{125}I-NGF incubation. Axons were rinsed with cold PBS for 20-30 s (or in some experiments not rinsed), and then either: i) fresh medium containing 1 μg/ml NGF (except 1 experiment, using 0.4 μg/ml) was added back to the compartments in dissociation experiments, or; ii) the axons were immediately harvested to determine axon-associated ^{125}I-NGF. Axons were all eventually harvested by vigorous trituration with only water which reduced the release of nonspecifically-bound ^{125}I-NGF from the collagen substrate, compared to detergent extraction. The 20-30 s rinse prior to axon harvesting or chasing was found in a separate experiment not to reduce levels of axon-associated ^{125}I-NGF, compared to no rinse. Also there was no correlation between rinsing and relative transport rates, which also implied that significant amounts of ^{125}I-NGF did not dissociate from low affinity receptors on axons during a 20-30 s rinse.

Specific transport, axon association, and dissociation were determined by subtracting nonspecific values from totals. Nonspecific values were determined by performing the same procedures in cultures in which a 100-200-fold higher concentration of unlabeled NGF was included in the ^{125}I-NGF incubation with distal axons. Typically, nonspecific transport accounted for less than 10% of total transport, while nonspecific association accounted for 40-50% of the total axon association. The decrease in free ^{125}I-NGF concentration in axon compartments due to 24 hr of axonal uptake and transport was at most 22%, but was most often less than 10%. Cell body/proximal axon ^{125}I-NGF at steady-state ranged from 1-96 pg, but it is not informative to make comparisons of absolute quantities between experiments because of the variability in neuronal number and axon growth between culture platings.

Kinetic Analysis of Degradation

Kinetic analysis was performed on the decay of cell-associated radioactivity. The pulse-chase protocol is described under Results. Cultures were used in which distal axons were axotomized at the end of the pulse, which eliminated interference due to the prolonged chase-transport from distal axons. Cell-associated radioactivity at the various intervals was not measured in these cultures, but instead was estimated by subtracting the cumulative release at each time point from the total amount of radioactivity released during the chase. This method could be used because the total, cumulative release of radioactivity over at least 30 hr was a close estimate of the initial cell-associated NGF. That is, in cultures in which distal axons were left intact during the chase the amount of radioactivity measured in cell bodies/proximal axons at the end of a 30 hr chase represented only 5% of the total, center-compartment radioactivity (medium + cell extract), some of which was due to nonspecific transport. In cultures where chase-transport was absent, residual cell body NGF was likely only about 2-3%. The estimated fraction of cell-associated ^{125}I-NGF was then plotted as a function of time.

RESULTS

NGF Is Mostly Associated With Distal Axons at Steady-State

The steady-state distribution of NGF in 3-compartment cultures (shown in Fig. 4.1*a*) was investigated by bathing distal axons continuously in ^{125}I-NGF for 15-24 hr, during which time some of the ^{125}I-NGF was retrogradely transported to cell bodies and proximal axons. Previous results have shown that steady-state association with axons is reached within 2 hr (Hawrot, 1982), and steady-state accumulation of ^{125}I-NGF in cell bodies/proximal axons is reached in about 8 hr (Claude et al., 1982b). Also, most radioactivity in cell bodies/proximal axons is intact ^{125}I-NGF by SDS-PAGE analysis (Ure and Campenot, 1994). As shown in Figure 4.2, the ^{125}I-NGF which retrogradely accumulated in cell bodies/proximal axons at steady state accounted for only 5-30% of the cellular ^{125}I-NGF, while the remainder was associated with distal axons. The distribution was slightly age-dependent, such that proportionally more ^{125}I-NGF became axon-associated in older cultures. Interestingly, the distribution was only weakly dependent on ^{125}I-NGF concentration, even though the concentration of ^{125}I-NGF supplied to distal axons ranged from 0.2-40 ng/ml (8 pM - 1.5 nM). The highest cell body accumulation (30%) occurred in an experiment using 0.2 ng/ml ^{125}I-NGF, but in 2 other experiments with this concentration the cell body accumulation was not markedly higher than at higher ^{125}I-NGF concentrations. At 8 pM ^{125}I-NGF, most ^{125}I-NGF binding should be to high affinity receptors, based on reported receptor affinities from other neurons (Sutter et al., 1979; Godfrey and Shooter, 1986). Therefore, at this concentration the abundance of ^{125}I-NGF associated with distal axons could not have resulted from extensive binding to low affinity receptors that might not have participated in transport.

Axon-Associated ^{125}I-NGF Is Mostly Surface-Bound

Dissociation assays at 37°C were performed to determine whether the distal axon ^{125}I-NGF was surface-bound, or internalized and possibly en route to cell bodies. This method was used rather than acid-wash (Buxser et al., 1990) because the latter technique appeared to fix axons to the substrate. Following overnight incubations with 10-20 ng/ml ^{125}I-NGF, distal axons were chased at 37°C in the presence of ≥100-fold excess unlabeled NGF. All radioactivity released from axons was intact ^{125}I-NGF by SDS-PAGE analysis (not shown). The warm chase was performed over 2 consecutive intervals of 6 and 18 hr. Figure 4.3 shows representative results from 1 of 4 experiments. From all experiments, over the total 24 hr chase 75-97% (mean=85%) of the axon-associated ^{125}I-NGF was released, in similarity to other cells (Sutter et al., 1979; Landreth and Shooter, 1980). Most of the release occurred during the first chase interval, but about 20% occurred later, indicative of very slow dissociation of some of the ^{125}I-NGF. There was no indication that ^{125}I-NGF release varied with culture age (16-42-day-old cultures).

As has been suggested previously for PC12 cells (Eveleth and Bradshaw, 1988; Kasaian and Neet, 1988; Buxser et al., 1990), some of the warm-chased ^{125}I-NGF may have been released from an intracellular pool by retroendocytosis. Since retroendocytosis has been shown to be blocked by dinitrophenol (DNP) treatment (Marshall, 1985; Formisano et al., 1994), we included a group with DNP (0.5 mM) in the medium during the first 6 hr chase. DNP, which depletes ATP stores, is effective in our culture model in blocking retrograde transport of leukemia inhibitory factor (Ure et al., 1994). Three experiments were performed using 10-20 ng/ml ^{125}I-NGF, and a representative result is shown in Figure 4.3. DNP treatment did not reduce the amount of ^{125}I-NGF released from the axons and even slightly increased its release in 2 of 3 experiments, in similarity to previous reports (Olender and Stach, 1980, 1981; Stach and Wagner, 1982). These results suggest that axons did not retroendocytose ^{125}I-NGF, and in turn suggest that approximately 85% of distal axon NGF was surface-bound, a portion of which was associated with very slowly-dissociating (high affinity) sites, in similarity to a previous report (Godfrey and Shooter, 1986).

Rate of Retrograde Transport

To directly quantify the rate at which the axon-associated ^{125}I-NGF was delivered to cell bodies/proximal axons, additional data were used from the experiments described in Fig. 4.2. Total NGF transport was calculated as the combined radioactivity collected from the cell bodies/proximal axons, representing intact NGF, and from the medium bathing them, representing products of NGF degradation released by the neurons during the incubation (Ure and Campenot, 1994). After 24 hr, more than 85% of the transported ^{125}I-NGF was degraded and released into the medium. The average transport rate was determined firstly by dividing total NGF transport by the period of transport which averaged 20 hr (cpm/hr), and then expressing it as a percentage of NGF associated with distal axons (final units = %/hr). The rate determined by this method differed by ≤2%/hr compared to the rate calculated once steady-state transport was reached. This small discrepancy likely reflected the absence of ^{125}I-NGF transport within the first hour of ^{125}I-NGF application to distal axons (Claude et al., 1982b). When ^{125}I-NGF was supplied to distal axons for 15-24 hr, only 2-25% of the distal axon ^{125}I-NGF was transported to cell bodies each hour, with the rate declining as a function of culture age (Fig. 4.4). Expressed differently, no less than 4 hr was required for ^{125}I-NGF to be transported in an amount equivalent to the amount associated with axons at steady-state, and in older cultures as long as 50 hr was required. The transport rate was not strongly dependent on the concentration of ^{125}I-NGF supplied to axons, which ranged from 0.2-40 ng/ml (8 pM - 1.5 nM). For example, in 2 experiments the transport rates in 4 ng/ml ^{125}I-NGF (0.15 nM) were only 1%/hr higher than in 40 ng/ml ^{125}I-NGF (1.5 nM). Using 0.2 ng/ml ^{125}I-NGF, at which the binding should be predominantly to high affinity receptors, we found that the transport rate in only 1 of 3 experiments differed markedly from transport rates at higher ^{125}I-NGF concentrations. These results are consistent with receptors of multiple affinities participating in NGF transport and suggest that transport occurred at a low rate even when ^{125}I-NGF associated mostly with high affinity receptors.

NGF is Retrogradely Transported At A Velocity of 10-20 mm/hr

We have used the release of degraded ^{125}I-NGF from the cell bodies to estimate the velocity of NGF retrograde transport. This was accomplished by comparing the time course of release in 3-compartment cultures with the time course in 5-compartment cultures in which the NGF was transported an additional 5 mm through an intermediate axon compartment (Figures 4.1*a* and 4.1*b*). We assumed that internalization and loading of NGF into the transport system as well as degradation and release of NGF from cell bodies occurred with the same time course in both types of cultures. Therefore, any delay in the appearance of degraded NGF from cell bodies in 5-compartment cultures would represent the time required for transport through the extra 5 mm of axon. After applying ^{125}I-NGF to distal axons, medium in the center compartments was exchanged at 15-min intervals. We observed that the first release of radioactivity from cell bodies was delayed in 5-compartment cultures by only 15-30 min (1-2 intervals), compared to 3-compartment cultures (Fig. 4.5). Therefore, the transport velocity is estimated to have been 10-20 mm/hr (i.e. 5 mm/15-30 min). This is higher than the estimate of 2-3 mm/hr made for NGF retrograde transport in adult rat sympathetic neurons in vivo (Hendry et al., 1974a,b; Johnson et al., 1978), but is similar to estimates of 7-13 mm/hr for NGF transport by sensory neurons (Stoeckel et al., 1975; Yip and Johnson, 1986) and 12 mm/hr for dopamine β-hydroxylase transport in sympathetic axons of the sciatic nerve (Brimijoin and Helland, 1976).

Little or No ^{125}I-NGF is Degraded or Released From Axons During Retrograde Transport

Some NGF in sympathetic axons (Claude et al, 1982a) and in neurites of PC12 cells (Bernd and Greene, 1983) is associated with lysosomal organelles. Moreover, a variety of evidence supports the possibility that degradative events might occur in axons (Broadwell, 1980; Gatzinsky et al, 1991a,b; Doherty et al., 1990; Renfrew and Hubbard, 1991; Overly et al., 1995). Also, release of previously internalized, intact ^{125}I-NGF from PC12 cells has been reported (Eveleth and Bradshaw, 1988; Buxser et al., 1990). We addressed the possibility of en route release of breakdown products or intact NGF by investigating transport in five-

compartment cultures shown in Figure 4.1c. In these cultures there are additional compartments interposed between the cell body/proximal axon compartment and the distal axon compartment where ^{125}I-NGF was applied. One of these compartments is large and is termed the intermediate axon compartment. We were able to determine whether any NGF being transported through the intermediate axon compartment was released en route to cell bodies. ^{125}I-NGF at 10-50 ng/ml was applied to distal axons (Compartments #4 & #5) for 22-40 hr, after which all of the radioactivity transported into the cell body/proximal axon and intermediate axon compartments (#1 and #2) was quantified. Mean results from 13 cultures are shown in Figure 4.6. Of all the radioactivity collected, only 3% was from the medium bathing intermediate axons, which was not significantly above background. Thus, little or no NGF or its breakdown products were released from axons while NGF was en route to cell bodies. In previous experiments we showed that cell bodies/proximal axons do not retain low molecular weight breakdown products of ^{125}I-NGF which indicates that they are released quickly from the neurons (Ure and Campenot, 1994). Thus, it is unlikely that in the present experiments ^{125}I-NGF was degraded in intermediate axons and that the degraded ^{125}I-NGF was transported to cell bodies/proximal axons. Therefore, these results suggest that nearly all ^{125}I-NGF loaded onto the retrograde transport system was delivered intact to cell bodies.

NGF Degradation Following Retrograde Transport

The turnover of cell body/proximal axon NGF was investigated in 3-compartment cultures, both under steady-state conditions and in pulse-chase experiments. Under steady-state conditions, radioactivity levels in cell bodies/proximal axons are relatively constant beyond approximately 10 hr (Claude et al., 1982b) and degraded ^{125}I-NGF is released into the medium at a linear rate (not shown). Turnover rates can be determined by sampling the radioactivity released into the medium during this time and comparing the release to ^{125}I-NGF levels in the cell bodies/proximal axons upon harvesting. From 10, 3-compartment cultures (14-38 days old) we found, on average, that the release of degraded ^{125}I-NGF was equivalent to 39% of the cell body/proximal axon pool per hour, corresponding to an average turnover interval of 2.7 hr (range 1.6 - 4.3 hr).

For the pulse-chase analysis of degradation rates, ^{125}I-NGF (50-200 ng/ml) was supplied to distal axons in 26-36-day-old cultures for 5 hr during which ^{125}I-NGF accumulated both in distal axons and in cell bodies/proximal axons. The ^{125}I-NGF-containing medium was then exchanged with medium containing at least 200 ng/ml NGF. During the chase the medium bathing cell bodies/proximal axons was exchanged several times and the released radioactivity quantified. Since this radioactivity represents only degraded ^{125}I-NGF (Ure and Campenot, 1994), we were able to determine the time course of release of ^{125}I-NGF degradation products. Representative results from 1 of 4 experiments are shown in Figure 4.7a (chase-intact cultures). The degradation and release of ^{125}I-NGF breakdown products occurred with a half-life of 6.1 hr, which reflects not only the clearance of ^{125}I-NGF which was in cell bodies at the end of the pulse, but also reflects retrograde transport and clearance of ^{125}I-NGF associated with distal axons at the end of the pulse.

The pulse-chase analysis was also performed in cultures in which distal axons were removed immediately following the 5-hr ^{125}I-NGF pulse (chase-axotomized cultures). Since there was no prolonged retrograde transport in these cultures, the accumulation of radioactivity in medium bathing cell bodies/proximal axons during the chase largely reflected degradation of the ^{125}I-NGF which resided in cell bodies at the end of the pulse. A representative time course, from 1 of 3 experiments, is shown in Figure 4.7a. As expected, the half-maximal release of ^{125}I-NGF degradation products in the chase-axotomized cultures occurred sooner ($t_{1/2}$ = 3 hr) than in cultures in which distal axons were left intact during the chase ($t_{1/2}$ = 6.1 hr), although the pattern of clearance was similar in both types of cultures. From the 3 hr difference in half-maximal release we can conclude that when the ^{125}I-NGF supply is removed from distal axons (in chase-intact cultures) and the axons are chased with NGF, significant amounts of ^{125}I-NGF continue to be retrogradely transported for no more than about 3 hr. Following its delivery to cell bodies, ^{125}I-NGF is then degraded with an average half-life of approximately 3 hr.

To determine if all ^{125}I-NGF in cell bodies/proximal axons was degraded at a uniform rate, or alternatively if there was more than one rate of degradation, we performed a kinetic analysis (see Materials and Methods) of the data from the chase-axotomized cultures above. Results from all 3 experiments (total of 9 cultures) are shown in Figure 4.7b. In each experiment the decay in cell body ^{125}I-NGF was biphasic, suggesting that there were 2 distinct rates of ^{125}I-NGF degradation. Slopes from the early and late stages of ^{125}I-NGF turnover appeared to differ

2-3-fold. These data suggest that cell bodies/proximal axons contained 2 or more functionally different pools of ^{125}I-NGF that were degraded at different rates, the net result being an average half-life of 3 hr.

DISCUSSION

Some Cellular Events Promote NGF Accumulation in Cell Bodies

Retrograde accumulation of trophic factor in cell bodies is believed to be important for retrograde signaling. Several aspects of the processing of NGF by sympathetic neurons which we have investigated promote accumulation of NGF in cell bodies. Firstly, we observed that little or no NGF was degraded or released intact from axons during retrograde transport, which was not known from previous experiments. Degradation or release of intact NGF during transport, if it had been observed, would have seriously questioned views about the role of NGF transport.

A second aspect is the rapid transport velocity for NGF of 10-20 mm/hr. This velocity matches reported retrograde organelle velocities from a variety of axons (Forman et al., 1977; Smith and Cooper, 1981; Koles et al., 1982; Breuer et al., 1987; Abbate et al., 1991), which suggests that once NGF is loaded onto the transport mechanism it is optimally delivered to cell bodies. The several-fold, higher velocity which we observed, as compared to that in sympathetic neurons in vivo (Hendry et al., 1974a,b; Johnson et al., 1978), might suggest that the velocity of retrograde transport is faster in immature neurons used for culturing than in adult neurons used in the in vivo studies.

A third process favoring accumulation of NGF in cell bodies is a relatively slow degradation rate. As compared to the turnover of a wide variety of internalized ligands in nonneuronal cells, the 3 hr half-life for cell body NGF which we observed is relatively slow (Chen et al., 1982; Huang et al., 1982; Wakai et al., 1984; Davies et al., 1985; Fujii et al., 1986; Zoon et al., 1986; Roupas and Herington, 1987; Sorkin et al., 1991; Nielson, 1992; Yanai et al., 1991; Auletta et al., 1992; Pandey, 1992; Zapf et al., 1994). Interestingly, kinetic analysis suggested that the 3 hr average half-life is likely to be the net result of 2 distinct rates of degradation. Basic FGF and TNF-α can be internalized and degraded at different rates depending on the type of receptor to which they are bound (Pennica et al., 1992; Gleizes et al., 1995), so by analogy, perhaps the degradation rate for NGF is different depending on whether NGF is bound to trkA or p75.

NGF Transport is Rate-Limited Upstream of the Transport Mechanism

We observed that no less than 4 hr was required for ^{125}I-NGF to be retrogradely transported in amounts equivalent to steady-state levels in distal axons. This interval corresponds to a maximum transport rate of 25%/hr. Periods of at least 10 hr (transport rate ≥10%/hr) were most common. Transport rates appeared to be only weakly dependent on the ^{125}I-NGF concentration, which ranged 200-fold from 8 pM - 1.5 nM, suggesting that receptors of more than one affinity were involved in NGF transport. This conclusion is consistent with previous findings in vivo (Dumas et al,.1979). Since virtually all axonal binding should be to high affinity receptors at 8 pM NGF, based on binding characteristics of other neurons (Sutter et al., 1979; Godfrey and Shooter, 1986), and since it has been previously shown that NGF bound to high affinity receptors in PC12 cells is internalized at a high rate (Bernd and Greene, 1984), we expected to observe higher transport rates than we did. The low transport rate observed at any concentration used did not reflect a low capacity of the transport mechanism, since previous results indicate that retrograde transport does not saturate until at least 4 nM NGF (Hawrot, 1982). The low transport rate also did not result from release of degraded or intact NGF while it was on the transport mechanism, as shown above. Therefore, we conclude that retrograde delivery of NGF to cell bodies was rate-limited prior to the shuttling of NGF-containing organelles along the microtubule-based transport mechanism. The finding that approximately 85% of the distal axon NGF was surface-bound at steady-state strongly suggests that internalization was rate-limiting. Theoretically, had the rate-limiting step occurred following internalization, then a large intra-axonal accumulation of NGF should have been observed.

We considered whether the transport rate was low because of a high fraction of binding to low affinity receptors which might not have been efficient at internalizing NGF (Bernd and Greene, 1984; Hosang and Shooter, 1987; Kasaian and Neet, 1988). This is an inadequate explanation for the transport rates observed at 8 pM NGF, since at this concentration very little of the binding should have been to low affinity receptors. Since p75 neurotrophin receptor binds NGF with mostly low affinity, the low transport rate at 8 pM NGF can not be explained by excessive binding to p75. It is possible, however, that the slightly lower transport rates which were observed when using the highest NGF concentration (40 ng/ml) might have resulted from

increased binding to p75, since several studies indicate that p75 internalizes and/or transports NGF at a low rate, if at all (Le Bivic et al., 1991; Kahle and Hertel, 1992; Mahadeo et al., 1994; Kahle et al.,1994; Curtis et al., 1995). Furthermore, augmented p75 binding might have been the reason for lower transport rates in older cultures. We have found that p75 mRNA levels increase with culture age in neurons given a concentration of NGF similar to that used to maintain cultures in the present study (Ma et al., 1992).

As an explanation for the low transport rate, we hypothesize that a large fraction of the axon-bound NGF might have been bound to receptors that did not have immediate access to sites of internalization. That is, perhaps molecules necessary for efficient internalization are in limited supply or are compartmentalized in axons. For example, although it has been shown that receptors along sensory axons in vivo do internalize and retrogradely transport NGF (Richardson and Riopelle, 1984), it is possible that only receptors on growth cones internalize NGF at a high rate.

Transport Velocity Does Not Account for the Delay in NGF Transport

^{125}I-NGF first appears in cell bodies/proximal axons after a delay of approximately 1 hr following its application to distal axons (Claude et al., 1982b; additional data not shown). This delay is not likely to result from a delay in ^{125}I-NGF binding to receptors on the axonal surface, since signficant binding to distal axons occurs within 10 min of ^{125}I-NGF application (Hawrot, 1982). We can now consider whether transport velocity accounts for the delay. Since the transport velocity was found to be 10-20 mm/hr, and since a distance of only 1 mm separates distal axon and cell body/proximal axon compartments, ^{125}I-NGF should have appeared in cell bodies/proximal axons within several minutes if it was immediately internalized in axons and loaded onto the retrograde transport mechanism. Moreover, the transport mechanism should have been sufficiently rapid to transport a large quantity of ^{125}I-NGF (e.g. 1 axonal receptor load) within the first hour of incubation, since the longest distance over which ^{125}I-NGF had to be transported in, for example, 20-day-old cultures was about 20 mm, based on observed axon extension rates of 1 mm/day (Campenot, 1982a). Assuming that the average transport rate measured after 4 hr of transport (data not shown; see also Claude et al., 1982b) is similar to rates observed during earlier periods, our assays should have detected transport within several minutes.

Therefore, we can now interpret that the lag in transport is not a reflection of the transport velocity. Instead, the lag might indicate that NGF-receptor complexes reside at the axon surface for a considerable period before being internalized, which is consistent with our observation of a large proportion of axonal ^{125}I-NGF at the surface. Also, NGF-containing organelles could possibly undergo a maturation step prior to loading onto the retrograde transport mechanism, which could contribute to the lag. Endosomal maturation has been described for EGF (Dunn and Hubbard, 1984; Schmid et al., 1988; Stoorvogel et al., 1991).

Distal Axons as Reservoirs of NGF

A surprising result from this study was that less NGF was maintained in cell bodies by retrograde transport than was associated with distal axons at steady-state. Given the importance of retrograde signaling to neurons and the important role that trophic transport is thought to play in this process, it could have been predicted that more ^{125}I-NGF would have accumulated in cell bodies. A relatively low level of cell body NGF is not an obvious interpretation from previous studies in vivo showing that NGF is more concentrated in sympathetic ganglia than in samples of target tissues (Korsching and Thoenen, 1983a, 1988; Nagata et al., 1987). However, in similarity to our findings, more recent studies in vivo report that at least for some sympathetic neurons NGF is extensively associated with axon terminals (Liu et al., 1996). As already discussed, several transport parameters are consistent with a role for NGF transport in retrograde signaling, but the larger amount of NGF associated with distal axons than in cell bodies suggests other important functional roles. Axonal NGF undoubtedly has local signaling functions such as promoting axon growth, but additionally, axonal NGF might generate other types of retrograde signals which travel to cell bodies unaccompanied by NGF.

REFERENCES

Abbate SL, Atkinson MB, Breuer AC (1991) Amount and speed of fast axonal transport in diabetes. Diabetes 40:111-117.

Auletta M, Nielsen FC, Gammeltoft S (1992) Receptor-mediated endocytosis and degradation of insulin-like growth factor I and II in neonatal rat astrocytes. J Neurosci Res 31:14-20.

Bernd P, Greene LA (1983) Electron microscopic radioautographic localization of iodinated nerve growth factor bound to and internalized by PC12 cells. J Neurosci 3:631-643.

Bernd P, Greene L (1984) Association of ^{125}I-nerve growth factor with PC12 pheochromocytoma cells. J Biol Chem 259:15509-15516.

Breuer AC, Lynn MP, Atkinson MB, Chou SM, Wilbourn AJ, Marks KE, Culver JE, Fleegler EJ (1987) Fast axonal transport in amyotrophic lateral sclerosis: An intra-axonal organelle traffic analysis. Neurol 37:738-747.

Brimijoin S, Helland L (1976) Rapid retrograde transport of dopamine-ß-hydroxylase as examined by the stop-flow technique. Br Res 102:217-228.

Broadwell RD (1980) Cytochemical localization of acid hydrolases in neurons of the mammalian central nervous system. J Histochem Cytochem 28:87-89.

Buxser S, Decker D, Ruppel P (1990) Relationship among types of nerve growth factor receptors on PC12 cells. J Biol Chem 265:12701-12710.

Campenot RB (1977) Local control of neurite development by nerve growth factor. Proc Natl Acad Sci USA 74:4516-4519.

Campenot RB (1982a) Development of sympathetic neurons in compartmentalized cultures. I. Local control of neurite growth by nerve growth factor. Dev Biol 93:1-12.

Campenot RB (1982b) Development of sympathetic neurons in compartmentalized cultures. II. Local control of neurite survival by nerve growth factor. Dev Biol 93:13-21.

Campenot RB (1992) Construction and use of compartmented cultures. In: Protocols for neural cell culture. (Federoff S, Richardson A, eds), pp 53-63. New Jersey: Humana.

Campenot RB (1994) NGF and the local control of nerve terminal growth. J Neurobiol 25:599-611.

Campenot RB, Walji AH, Draker DD (1991) Effects of sphingosine, staurosporine, and phorbol ester on neurites of rat sympathetic neurons growing in compartmented cultures. J Neurosci 11:1126-1139.

Chen BD-M, Hsu S, Lin H-S (1982) Binding, internalization, and degradation of colony stimulating factor by peritoneal exudate macrophages. Biochim Biophys Acta 721:366-373.

Claude P, Hawrot E, Parada I (1982a) Ultrastructural studies on the intracellular fate of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J Cell Biochem 20:1-13.

Claude P, Hawrot E, Dunis DA, Campenot RB (1982b) Binding, internalization, and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J Neurosci 2:431-442.

Curtis R, Adryan KM, Stark JL, Park J, Compton DL, Weskamp G, Huber LJ, Chao MV, Jaenisch R, Lee KF, Lindsay RM, DiStefano PS (1995) Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14:1201-1211.

Davies PF, Truskey GA, Warren HB, O'Connor SE, Eisenhaure BH (1985) Metabolic cooperation between vascular endothelial cells and smooth muscle cells in co-culture: Changes in low density lipoprotein metabolism. J Cell Biol 101:871-879.

Doherty JJ, Kay DG, Lai WH, Posner BI, Bergeron JJM (1990) Selective degradation of insulin within rat liver endosomes. J Cell Biol 110:35-42.

Dumas M, Schwab ME, Thoenen H (1979) Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. J Neurobiol 10:179-197.

Dunn WA, Hubbard AL (1984) Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: Ligand and receptor dynamics. J Cell Biol 98:2148-2159.

Ehrhard PB, Otten U (1994) Posnatal ontogeny of the neurotrophin receptors trk and trkB mRNA in rat sensory and sympathetic ganglia. Neurosci Letts 166:207-210.

Eveleth DD, Bradshaw RA (1988) Internalization and cycling of nerve growth factor in PC12 cells: Interconversion of Type II (Fast) and Type I (Slow) nerve growth factor receptors. Neuron 1:929-936.

Forman DS, Padjen AL, Siggins GR (1977) Effect of temperature on the rapid retrograde transport of microscopically visible intra-axonal organelles. Br Res 136:215-226.

Formisano P, DeNovellis G, Miele C, Tripodi F, Caruso M, Palumbo G, Beguinot L, Beguinot F (1994) Internalization of the constitutively active arginine 1152-glutamine insulin receptor occurs independently of insulin at an accelerated rate. J Biol Chem 269:16242-16246.

Fujii M, Sugamura K, Sano K, Nakai M, Sugita K, Hinuma Y (1986) High-affinity receptor-mediated internalization and degradation of interleukin 2 in human T cells. J Exp Med 163:550-562.

Gatzinsky KP, Berthold C-H, Fabricius C, Mellström A (1991a) Lysosomal activity at nodes of Ranvier in dorsal column and dorsal root axons of the cat after injection of horseradish peroxidase in the dorsal column nuclei. Br Res 566:131-139.

Gatzinsky KP, Berthold C-H, Fabricius C (1991b) Lysosomal activity in developing cat alpha-motor axons under normal conditions and during retrograde axonal transport of horseradish peroxidase. J Comp Neurol 312:599-609.

Gleizes P-E, Noaillac-Depeyre J, Amalric F, Gas N (1995) Basic fibroblast growth factor (FGF-2) internalization through the heparan sulfate proteoglycans-mediated pathway: an ultrastructural approach. Eur J Cell Biol 66:47-59.

Godfrey EW, Shooter EM (1986) Nerve growth factor receptors on chick embryo sympathetic ganglion cells: Binding characteristics and development. J Neurosci 6:2543-2550.

Goedert M, Stoeckel K, Otten U (1981) Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons. Proc Natl Acad Sci USA 78:5895-5898.

Hawrot E (1982) Characteristics of the association of nerve growth factor with primary cultures of rat sympathetic neurons. J Neurosci Res 8:213-224.

Hendry IA, Stockel K, Thoenen H, Iversen LL (1974a) The retrograde axonal transport of nerve growth factor. Br Res 68:103-121.

Hendry IA, Stach R, Herrup K (1974b) Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. Br Res 82:117-128.

Hogue-Angeletti R, Stieber A, Gonatas NK (1982) Endocytosis of nerve growth factor by PC12 cells studied by quantitative ultrstructural autoradiography. Br Res 241:145-156.

Hosang M, Shooter EM (1987) The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. EMBO J 6:1197-1202.

Huang JS, Huang SS, Kennedy B, Deuel TF (1982) Platelet-derived growth factor. Specific binding to target cells. J Biol Chem 257:8130-8136.

Johnson EM Jr, Andres RY, Bradshaw RA (1978) Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I]NGF. Br Res 150:319-331.

Kahle P, Hertel C (1992) Nerve growth factor (NGF) receptor on rat glial cell lines. Evidence for NGF internalization via $p75^{NGFR}$. J Biol Chem 267:13917-13923.

Kahle P, Barker PA, Shooter EM, Hertel C (1994) p75 nerve growth factor receptor modulates $p140^{trkA}$ kinase activity, but not ligand internalization, in PC12 cells. J Neurosci Res 38:599-606.

Kasaian MT, Neet KE (1988) Internalization of nerve growth factor by PC12 cells. J Biol Chem 263:5083-5090.

Kessler J, Black IB (1979) The role of axonal transport in the regulation of enzyme activity in sympathetic ganglia of adult rats. Br Res 171:415-424.

Koles ZJ, McLeod KD, Smith RS (1982) A study of the motion of organelles which undergo retrograde and anterograde rapid axonal transport in Xenopus. J Physiol 328:469-484.

Korsching S, Thoenen H (1983a) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Corrleation with density of sympathetic innervation. Proc Natl Acad Sci USA 80:3513-3516.

Korsching S, Thoenen H (1983b) Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. Neurosci Letts 39:1-4.

Korsching S, Thoenen H (1985) Treatment with 6-hydroxydopamine and colchicine decreases nerve growth factor levels in sympathetic ganglia and increases them in the corresponding target tissues. J Neurosci 5:1058-1061.

Korsching S, Thoenen H (1988) Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev Biol 126:40-46.

Landreth GE, Shooter EM (1980) Nerve growth factor receptors on PC12 cells: Ligand-induced conversion from low- to high-affinity states. Proc Natl Acad Sci USA 77:4751-4755.

Layer PG, Shooter EM (1983) Binding and degradation of nerve growth factor by PC12 phoechromocytoma cells. J Biol Chem 258:3012-3018.

Le Bivic A, Sambuy Y, Patzak A, Patil N, Chao M, Rodriguez-Boulan E (1991) An internal deletion in the cytoplasmic tail reverses the apical localization of human NGF receptor in transfected MDCK cells. J Cell Biol 115:607-618.

Liu, D. T., Reid, M. T., Bridges, D. C. M., and Rush, R. A. (1996). Denervation, but not ddecentralization, reduces nerve growth factor content of the mesenteric artery. J Neurochem. 66, 2295-2299.

Ma Y, Campenot RB, Miller FD (1992) Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117:135-141.

Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than $p140^{trk}$ binding. J Biol Chem 269:6884-6891.

Marshall S (1985) Dual pathways for the intracellular processing of insulin. J Biol Chem 260:13524-13531.

Nagata Y, Ando M, Takahama K, Iwata M, Hori S, Kato K (1987) Retrograde transport of endogenous nerve growth factor in superior cervical ganglion of adult rats. J Neurochem 49:296-302.

Nielsen S (1992) Time course and kinetics of proximal tubular processing of insulin. Am J Physiol 262:F813-F822.

Olender EJ, Stach RW (1980) Sequestration of ^{125}I-labeled ß nerve growth factor by sympathetic neurons. J Biol Chem 255:9338-9343.

Olender EJ, Stach RW (1981) Sequestration of ^{125}I-labeled ß nerve growth factor by embryonic sensory neurons. J Neurochem 37:436-442.

Overly CC, Lee K-D, Berthiaume E, Hollenbeck PJ (1995) Quantitative measurement of intraorganelle pH in the endosomal-lysosomal pathway in neurons by using ratiometric imaging with pyranine. Proc Natl Acad Sci USA 92:3156-3160.

Palmatier MA, Hartman BK, Johnson EM Jr (1984) Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. J Neurosci 4:751-756.

Pandey KN (1992) Kinetic analysis of internalization, recycling and redistribution of atrial natriuretic factor-receptor complex in cultured vascular smooth-muscle cells. Biochem J 288:55-61.

Paravicini U, Stoeckel K, Thoenen H (1975) Biological importance of retrograde axonal transport of nerve growth factor in adrenergic neurons. Br Res 84:279-291.

Pennica D, Lam VT, Mize NK, Weber RF, Lewis M, Fendly BM, Lipari MT, Goeddel DV (1992) Biochemical properties of the 75-kDa tumor necrosis factor receptor. J Biol Chem 267:21172-21178.

Renfrew CA, Hubbard AL (1991) Sequential processing of epidermal growth factor in early and late endosomes of rat liver. J Biol Chem 266:4348-4356.

Richardson PM, Riopelle RJ (1984) Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. J Neurosci 4:1683-1689.

Roupas P, Herington AC (1987) Receptor-mediated endocytosis and degradative processing of growth hormone by rat adipocytes in primary culture. Endocrinol 120:2158-2165.

Schmid SL, Fuchs R, Male P, Mellman I (1988) Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. Cell 52:73-83.

Smith RS, Cooper PD (1981) Variability and temperature dependence of the velocity of retrograde particle transport in myelinated axons. Can J Physiol Pharmacol 59:857-863.

Sorkin A, Waters C, Overholser KA, Carpenter G (1991) Multiple autophosphorylation site mutations of the epidermal growth factor receptor. Analysis of kinase activity and endocytosis. J Biol Chem 266:8355-8362.

Stach RW, Wagner, BJ (1982) Sequestration requirements for the degradation of ^{125}I-labeled β nerve growth factor bound to embryonic sensory neurons. J Neurosci Res 5:403-411.

Stöckel K, Thoenen H (1975) Retrograde axonal transport of nerve growth factor: specificity and biological importance. Br Res 85:337-341.

Stöckel K, Paravicini U, Thoenen H (1974) Specificity of the retrograde axonal transport of nerve growth factor. Br Res 76:413-421.

Stoeckel K, Schwab M, Thoenen H (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: A biochemical and morphological study. Br Res 89:1-14.

Stöckel K, Guroff G, Schwab M, Thoenen H (1976) The significance of retrograde axonal transport for the accumulation of systemically administered nerve growth factor (NGF) in the rat superior cervical ganglion. Br Res 109:271-284.

Stoorvogel W, Strous GJ, Geuze HJ, Oorschot V, Schwartz AL (1991) Late endosomes derive from early endosomes by maturation. Cell 65:417-427.

Sutter A, Riopelle RJ, Harrris-Warrick RM, Shooter EM (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J Biol Chem 254:5972-5982.

Ure DR, Campenot RB (1994) Leukemia inhibitory factor and nerve growth factor are retrogradely transported and processed by cultured rat sympathetic neurons. Dev Biol 162:339-347.

Wakai K, Tsushima T, Isozaki O, Sato Y, Sato K, Shizume K (1984) Processing of human growth hormone by rat hepatocytes in monolayer culture. Endocrinol 114:1475-1482.

Yanai S, Sugiyama Y, Kim DC, Iga T, Fuwa T, Hanano M (1991) Kinetic analysis of receptor-mediated endocytosis of epidermal growth factor by isolated rat hepatocytes. Am J Physiol 260:C457-C467.

Yip HK, Johnson EM Jr (1986) Comparative dynamics of retrograde transport of nerve growth factor and horseradish peroxidase in rat lumbar dorsal root ganglia. J Neurocytol 15:789-798.

Zapf A, Hsu D, Olefsky JM (1994) Comparison of the int racellular itineraries of insulin-like growth factor-I and insulin and their receptors in rat-1 fibroblasts. Endocrinol 134:2445-2452.

Zoon KC, Nedden DZ, Hu R, Arnheiter H (1986) Analysis of the steady-state binding, internalization, and degradation of human interferon-α_2. J Biol Chem 261:4993-4996.

FIGURES

Figure 4.1 Compartmented culture designs

Illustrations of individual tracks (20 tracks per culture) are shown from three different designs of compartmented cultures used in the present study. Teflon septa separate compartments. Distal axon compartments, where ^{125}I-NGF was always applied, were separated from cell body/proximal axon compartments by distances of: a, 1 mm; b, 6 mm, and; c, 8 mm. Illustrations are not to scale.

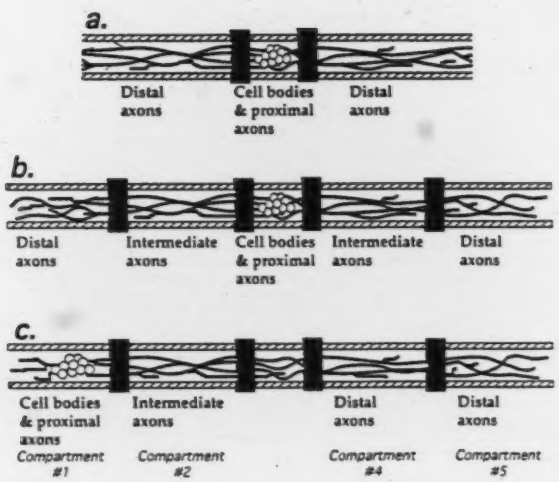

a.
Distal axons
Cell bodies & proximal axons
Distal axons
b.
Distal axons
Intermediate axons
Cell bodies & proximal axons
Intermediate axons
Distal axons
c.
Cell bodies & proximal axons
Compartment #1
Intermediate axons
Compartment #2
Distal axons
Compartment #4
Distal axons
Compartment #5

Figure 4.2 Relative distributions of cell-associated NGF

In 3-compartment cultures of various ages, 0.2-40 ng/ml ^{125}I-NGF (8 pM - 1.5 nM) was supplied to distal axons for 15-24 hr, during which time some ^{125}I-NGF was retrogradely transported to cell bodies/proximal axons. Distal axons in some cultures additionally received ≥100-fold excess NGF for determination of nonspecific association/transport. After removing the culture medium and rinsing all compartments, cell extracts were made and the radioactivity quantified. Shown are relative proportions of ^{125}I-NGF radioactivity associated with axons or accumulated in cell bodies/proximal axons, with nonspecific values subtracted. Concentrations of applied ^{125}I-NGF are: 0.2 ng/ml (*circles*); 3-20 ng/ml (*squares*); 40 ng/ml (*triangles*). Distal axon-associated ^{125}I-NGF is the sum of both side compartments. In each experiment 2-4 cultures were used per treatment group. Error bars (SEM) fall within symbols when not visible. Linear regressions were calculated from data from a total of 17 experiments.

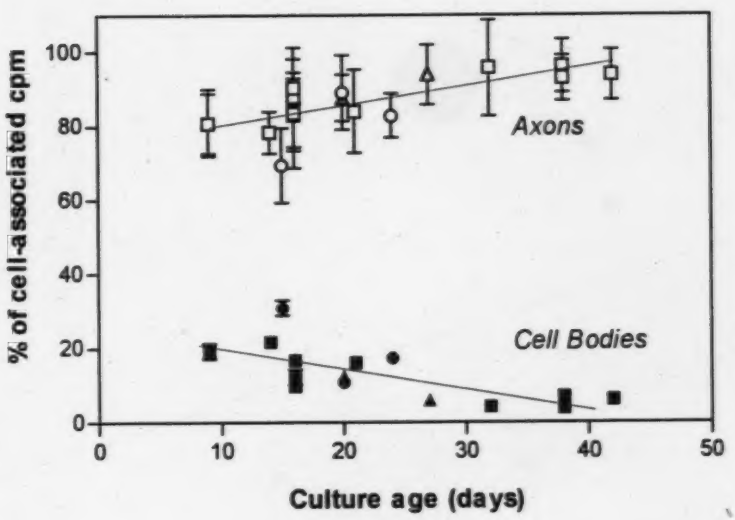

% of cell-associated cpm
100
80
60
40
20
0
0
10
20
30
40
50
Culture age (days)
Axons
Cell Bodies

Figure 4.3 ^{125}I-NGF dissociation from distal axons

In 37 day-old, 3-compartment cultures, distal axons were supplied 20 ng/ml ^{125}I-NGF for 10 hr, after which cultures were split into 3 groups: i) Distal axons were immediately harvested (6 sides); ii) Axonal ^{125}I-NGF was chased with 0.4 μg/ml NGF for 6 hr, then 18 hr, at 37°C (6 sides), and the dissociated ^{125}I-NGF collected following each chase; iii) Axonal ^{125}I-NGF was chased with 0.4 μg/ml NGF and 500 μM dinitrophenol (1000x dilution) for 6 hr at 37°C (4 sides) and the dissociated ^{125}I-NGF collected. Specific values for ^{125}I-NGF association and dissociation are shown (means ± SEM). Two other experiments gave similar results.

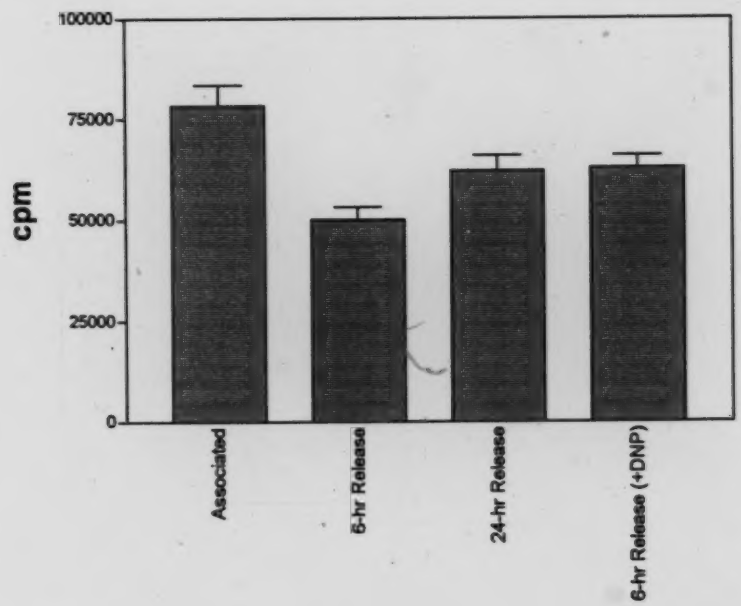

100000
75000
50000
25000
0
cpm
Associated
6-hr Release
24-hr Release
6-hr Release (+DNP)

Figure 4.4 Rates of retrograde transport

Data are taken from experiments described in Fig. 4.2. Transported ^{125}I-NGF was quantified by collecting all radioactivity from center compartments (medium + cell bodies/proximal axons) after the 15-24 hr incubations with 0.2-40 ng/ml ^{125}I-NGF. Total transport was divided by the transport interval and then compared to the amount of ^{125}I-NGF associated with distal axons at steady state (100%) to determine the transport rate (%/hr). Concentrations of applied ^{125}I-NGF are: 0.2 ng/ml (*circles*); 3-20 ng/ml (*squares*); 40 ng/ml (*triangles*). Error bars (SEM) fall within symbols when not visible. A linear regression was calculated from data from a total of 17 experiments.

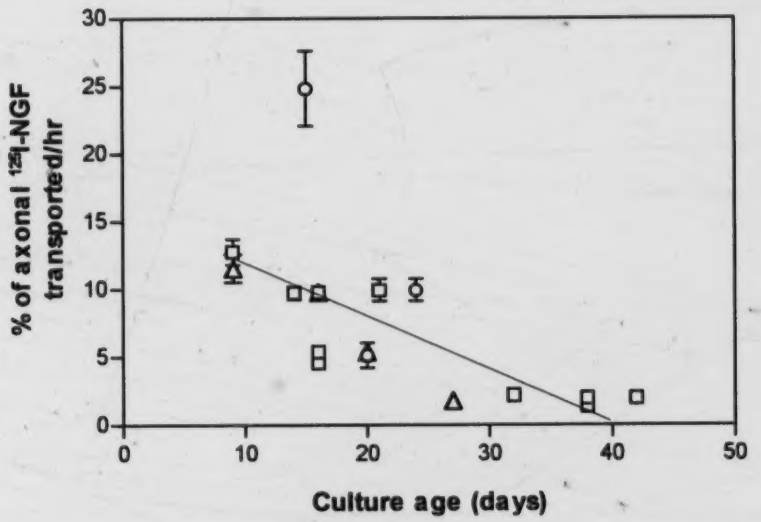

% of axonal ^{125}I-NGF transported/hr
30
25
20
15
10
5
0
0
10
20
30
40
50
Culture age (days)

Figure 4.5 Velocity of retrograde transport

In both 3-compartment and 5-compartment cultures (shown in Fig. 4.1a and 4.1b), distal axons were incubated with 10-65 ng/ml ^{125}I-NGF. At 15 min intervals, medium from the cell body/proximal axon compartment was exchanged and the radioactivity in the medium aliquots quantified. A, Representative cultures, in which the first appearance of radioactivity above background, representing release by the neurons is marked by an arrow. B, Cumulative data, showing when radioactivity was first released from cell bodies/proximal axons. The 15-30 min delay in 5-compartment cultures was attributed to transport across the intermediate axon compartment spanning 5 mm. Transport velocity was estimated as: 5 mm/(0.25 to 0.5 hr) = 10-20 mm/hr.

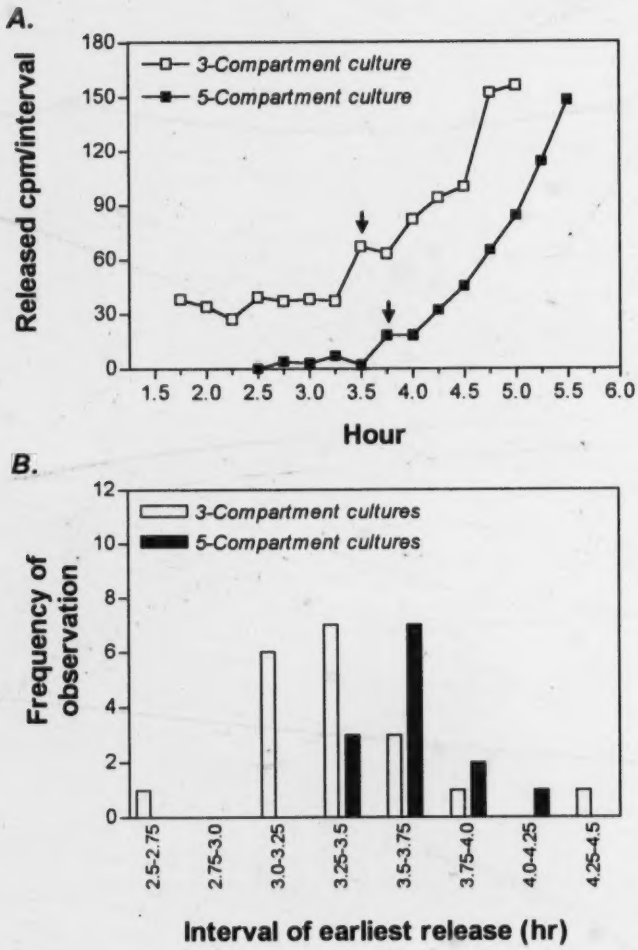

A.
Released cpm/interval
180
150
120
90
60
30
0
3-Compartment culture
5-Compartment culture
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Hour
B.
Frequency of observation
12
10
8
6
4
2
0
3-Compartment cultures
5-Compartment cultures
2.5-2.75
2.75-3.0
3.0-3.25
3.25-3.5
3.5-3.75
3.75-4.0
4.0-4.25
4.25-4.5
Interval of earliest release (hr)

Figure 4.6 Lack of release of NGF or its degradation products by intermediate axons

In 5-compartment cultures (shown in Fig. 4.1*c*), distal axons were incubated with 10-50 ng/ml ^{125}I-NGF for 22-40 hr, during which ^{125}I-NGF was retrogradely transported through intermediate axons and into cell bodies/proximal axons. Radioactivity was then collected from various fractions, as shown, Data are expressed as percentage of total, combined radioactivity from both compartments. Shown are means (± SEM) of 13 cultures.

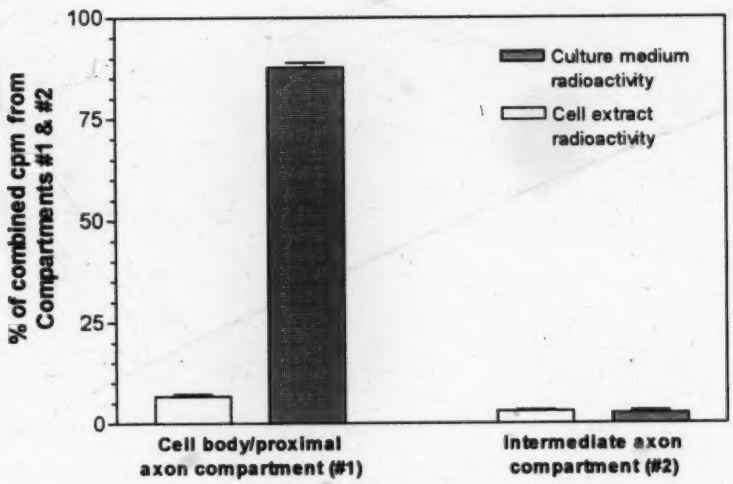
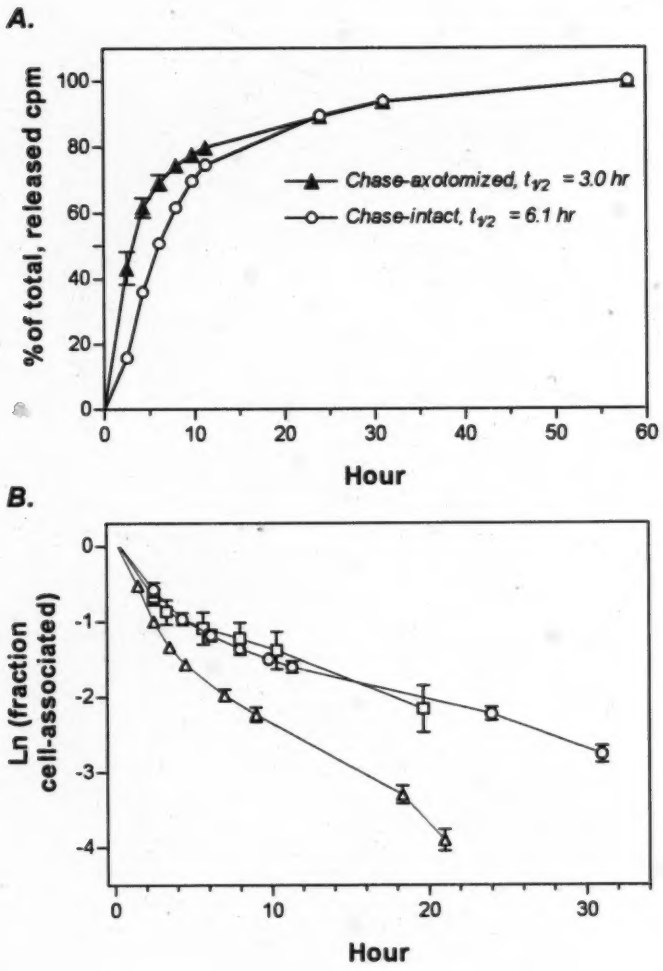

100
75
50
25
0
% of combined cpm from Compartments #1 & #2
Culture medium radioactivity
Cell extract radioactivity
Cell body/proximal axon compartment (#1)
Intermediate axon compartment (#2)

Figure 4.7 Time course of NGF degradation by pulse-chase analysis

A, Distal axons of 26-day-old, 3-compartment cultures were pulsed with 200 ng/ml ^{125}I-NGF for 5 hr, during which ^{125}I-NGF associated with distal axons and retrogradely accumulated in cell bodies/proximal axons. Following the pulse, distal axons were left intact in some cultures (circles) or were removed by axotomy in other cultures (triangles), and the ^{125}I-NGF-containing medium replaced with medium containing 200 ng/ml NGF. Radioactivity in the medium bathing cell bodies/proximal axons, representing ^{125}I-NGF degradation products, was then quantified repetitively in the same cultures by medium exchange at the times shown. Data are expressed as percentages of the total, cumulative release. Mean values from 1 of 3 experiments (± SEM, 3 cultures/group) are shown. B, Kinetic analysis of the estimated decay of ^{125}I-NGF from cell bodies/proximal axons from cultures in which distal axons were absent during the chase (see Materials and Methods). Results from 3 separate experiments, indicated by different symbols, are shown (9 cultures total).


A.
% of total, released cpm
100
80
60
40
20
0
0
10
20
30
40
50
60
Hour
Chase-exotomized, $t_{1/2}$ = 3.0 hr
Chase-intact, $t_{1/2}$ = 6.1 hr
B.
Ln (fraction cell-associated)
0
-1
-2
-3
-4
0
10
20
30
Hour

Chapter 5

Roles of NGF Receptors in ^{125}I-NGF Retrograde Transport by Rat Sympathetic Neurons in Compartmented Cultures

[Results presented in this chapter have not yet been submitted for publication.]

INTRODUCTION

The retrograde axonal transport of NGF is thought to be important for retrograde signaling by NGF. The first step in the retrograde delivery of NGF to cell bodies is the binding of NGF to receptors on axons. The two known NGF receptors are the trkA tyrosine kinase and the p75 neurotrophin receptor. trkA is a tyrosine kinase receptor which is sufficient to mediate the survival and growth-promoting effects of NGF (Bothwell, 1995). It has high specificity for binding NGF but has also been shown to bind neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4; Berkemeier et al., 1991; Ip et al., 1992; Dechant, 1993). trkA is the best candidate for delivering NGF and possibly the retrograde signal to cell bodies because most if not all tyrosine kinase receptors efficiently internalize their ligands (Sorkin and Waters, 1993). In support of this role, trkA has been shown to be retrogradely transported (Loy et al., 1994; Ehlers et al., 1995). Also, Ehlers et al. (1995) reported that retrogradely transported trkA in sensory axons from the sciatic nerve was tyrosine phosphorylated. Moreover, they found that when levels of free NGF in a sensory neuron target, the footpad, were varied either by injecting NGF or NGF anti-serum, the extent of trkA phosphorylation changed, consistent with the phosphorylation state of transported trkA being dependent on NGF binding more distally along axons.

The p75 receptor, which is structurally different from trkA, binds all neurotrophins with similar affinities (Rodriguez-Tébar et al., 1990, 1992; Timm et al., 1994). It is very frequently co-expressed with trkA in NGF-responsive neurons (Bothwell, 1995). The role of p75 in NGF transport, as well as in other aspects of neuron biology, remains controversial. Several studies investigating sympathetic neurons or other neurons have shown that p75 as well as the MC192 antibody to p75 are retrogradely transported (Taniuchi and Johnson, 1985: Taniuchi et al., 1986a; Johnson et al., 1987; Yan et al., 1988). Also in support of a role for p75, MC192, which increases ^{125}I-NGF association with p75 (Chandler et al., 1984), has been shown to increase ^{125}I-NGF transport (Taniuchi and Johnson, 1985). In contrast, Curtis et al. (1995) reported that p75

binding was not significantly involved in NGF transport by sympathetic neurons in adult rats and was minimally involved in NGF transport by sensory neurons. They found, however, that p75 was markedly involved in retrograde transport of BDNF, NT-3, and NT-4 by sensory neurons. In PC12 cells, which have been used extensively for studying NGF effects, p75 is reported not to participate in NGF internalization (Kahle et al., 1994). However, NGF is internalized in other cells where p75 but not trkA is expressed, albeit at relatively low levels (Le Bivic et al., 1991; Kahle and Hertel, 1992; Mahadeo et al., 1994).

Apart from the molecular distinctions of the NGF receptors, different receptors have been distinguished by kinetic and steady-state binding properties. From these analyses at least 2 binding sites have been documented in neurons (Sutter et al., 1979; Olender and Stach, 1980; Olender et al., 1981; Godfrey and Shooter, 1986; Meakin and Shooter, 1992). One has been called a low affinity or rapidly-dissociating (fast) receptor because it has a $K_D \approx 10^{-9}$ M and because NGF dissociates rapidly from this receptor at any temperature ($t_{1/2} < 1$ min). The other type has been called a high affinity or slowly-dissociating (slow) receptor because it binds NGF with a $K_D \approx 10^{-11}$ M and because NGF dissociates slowly from this receptor at 37°C ($t_{1/2} \geq 10$ min) and virtually not at all at low temperatures (e.g. 2°C). However, describing a single receptor type (e.g. high affinity/slow receptor) by either its binding affinity or its dissociation behaviour is not entirely accurate. For example, PC12 cells do not have receptors with 10^{-11} M affinities (high affinity), yet they do have slowly-dissociating receptors (Schecter and Bothwell, 1981; Bernd and Greene, 1984; Woodruff and Neet, 1986). Similarly, cells expressing trkA alone bind NGF mostly with low affinity, yet dissociation from trkA occurs only with slow kinetics (Kaplan et al., 1991; Klein et al., 1991; Jing et al., 1992; Meakin et al., 1992). Thus, slow dissociation is a necessary characteristic of high affinity binding, but slow receptors need not all bind NGF with high affinity but can also bind with low affinity. Slow dissociation is characteristic mostly of trkA, although under some conditions p75 has also been shown to represent a slow receptor with high affinity binding (Buxser et al., 1985; Bothwell, 1995). No studies have examined the role of slow receptors, per se, in retrograde transport, but the concentration-dependence of NGF transport in vivo has been previously examined (Dumas et al., 1979). In that study it was found that 2 distinct classes of receptors participated in NGF transport by sympathetic neurons in adult rats, with one receptor being of high affinity and one being of lower affinity. In PC12 cells only

high affinity receptors are reported to internalize NGF (Bernd and Greene, 1984; Hosang and Shooter, 1987).

In the present study we have examined the importance of different NGF receptors (slowly-dissociating, trkA, p75) in the retrograde transport of NGF by sympathetic neurons grown in compartmented cultures. p75 and trkA are the primary receptors in postnatal sympathetic neurons, although small quantities of trkC mRNA are also present (Wetmore and Olson, 1995). Their maximal survival and neurite extension in culture occurs in response to NGF, although NT-3 and NT-4 also exert these effects to a limited extent. BDNF does not support their survival or local axon growth (unpublished observations). We have firstly examined the role of slow receptors by comparing NGF binding to slow receptors on axons with the NGF transport rate. Secondly, we have examined the roles of trkA and p75 by measuring NGF association with axons and retrograde transport when binding to trkA or p75 was blocked by various treatments. Our results not only provide a quantitative account of contributions of the receptors to retrograde transport, but raise some possibilities about how the receptors, in particular p75, might function in NGF transport.

EXPERIMENTAL PROCEDURES

Materials

Newborn Sprague-Dawley rats were supplied by University of Alberta Farm. Trypsin was obtained from Calbiochem (La Jolla, CA). $L15CO_2$ culture medium was obtained from GIBCO Laboratories (Grand Island, NY). Adult rat serum was prepared from whole blood supplied by Lab Animal Services, University of Alberta. Rat tail collagen was prepared by the method of Hawrot and Patterson (1979). 2.5S NGF was purchased from Cedarlane Laboratories Ltd. (Hornby, Ontario). BDNF, NT-3, and NT-4 were kindly supplied by Regeneron Pharmaceuticals. Teflon dividers were purchased from Tyler Research Instruments (Edmonton, Alberta). $Na^{125}I$ was purchased from Amersham (Oakville, Ontario). PD-10 sephadex G-25M columns were purchased from Pharmacia Biotech (Baie d'Urfé, Quebec). Reagents whose suppliers are not stated were obtained from Sigma (St. Louis, MO). Labeled NGF and NT-3 supported survival and axon extension by sympathetic neurons. Labeled BDNF supported survival and axon extension by nodose neurons.

Compartmented Cultures

Sympathetic neurons were isolated from the superior cervical ganglia of newborn rats by 0.1% (w/v) trypsin incubation and mechanical dissociation, as previously described (Campenot et al., 1991). The standard culture medium was $L15CO_2$ supplemented with the prescribed additives and 6% methylcellulose. Compartmented cultures were constructed as previously described (Campenot, 1992). Briefly, rat tail collagen was air-dried onto 35 mm culture dishes and then parallel scratches made in the substrate. Teflon dividers (3-compartment design) were seated onto the collagen tracks using silicon vacuum grease. Cell suspension was plated into the center compartment of each culture (2000-3000 neurons/culture), and axons extended into left and right side compartments (distal axon compartments) containing NGF. For the first 6 days following plating, medium additives in the cell body compartment/proximal axon compartment included 2.5% rat serum, 1 mg/ml ascorbic acid, 10 ng/ml NGF, and 10 μM cytosine arabinoside. Thereafter cell body compartments received only rat serum and ascorbic acid. Axon

compartments received only NGF as a medium additive at a concentration of 100 ng/ml throughout the entire culture period. Medium was changed every 4-6 days. Cultures were maintained in a 5% CO_2 atmosphere at 37°C.

Radioiodination of Neurotrophins

NGF, BDNF, and NT-3 were radioiodinated by the lactoperoxidase method. The following ingredients were mixed at room temperature for 1 hr: 3-5 µl (1.5 mCi) $Na^{125}I$, 10 µl (10 µg) neurotrophin, 37 µl 0.5M potassium phosphate buffer (pH 7.4), 10 µl 33 µg/ml lactoperoxidase, and 10 µl 0.003% H_2O_2. After the first 30 min of incubation, an additional 10µl of H_2O_2 was added. The reaction was terminated with 5 µl ß-mercaptoethanol and 415 µl 1 mg/ml BSA in potassium phosphate buffer. Specific activities and labelling efficiencies were calculated after acid precipitation (20% trichloroacetic) of an aliquot of the reaction mixture. Mixtures were purified using Sephadex G-25M gel filtration columns. Radioactivity was quantified using a Wallac 1470 gamma counter. Specific activities averaged 146, 111, and 68 cpm/pg for NGF, BDNF, and NT-3, respectively. Ligands were used within 3 weeks of iodination. Molar concentrations of neurotrophins were based on a molecular weights of 26,000 g/mole.

Neurotrophin Retrograde Transport, Association, and Dissociation

The protocols for assaying neurotrophin transport, association, and dissociation began identically, by supplying radioiodinated neurotrophins, either alone or with additional treatments, to distal axons overnight, during which axon-association and retrograde acccumulation of neurotrophin in cell bodies reached steady-state. In all experiments one group of cultures were coincubated with iodinated neurotrophin plus a 100-200-fold excess of unlabeled neurotrophin. The neurotrophin transport, association, and dissociation that occurred in these cultures represented the nonspecific values for these variables. Unless otherwise stated, all measurements of ^{125}I-NGF transport, association, and dissociation given in this report represent specific values (i.e. nonspecific values subtracted).

Retrograde transport was quantified by measuring all radioactivity in cell bodies/proximal axons (collected by extraction with SDS-PAGE sample buffer) plus in the medium bathing them. The medium radioactivity almost entirely represented neurotrophin degradation products released by the cell bodies (Ure and Campenot, 1994). Following a 24 hr incubation with ^{125}I-NGF or ^{125}I-NT-3, approximately 85% of the transported radioactivity was in the medium fraction. Nonspecific ^{125}I-NGF transport represented less than 10% of the transport following incubation with ^{125}I-NGF alone.

To quantify neurotrophin association with distal axons, medium from the side compartments was removed thoroughly following the incubation and then an extract of the axons made with distilled water. This procedure was sufficient to remove all axonal material. Nonspecific association averaged 40% of the association following incubation with ^{125}I-NGF alone.

The procedure for determining the amount of ^{125}I-NGF bound to slowly-dissociating receptors is described in detail in Results. Nonspecific dissociation of ^{125}I-NGF from slow receptors averaged 35% of the dissociation in cultures incubated with ^{125}I-NGF alone during the association phase.

RESULTS

We used 3-compartment cultures in which newborn rat sympathetic neurons plated into a central (cell body/proximal axon) compartment extended axons into left and right, distal axon compartments. The culture model is described previously (Ure and Campenot, 1994). In these cultures ^{125}I-NGF supplied to distal compartments associates with distal axons and is retrogradely transported to cell bodies. Association to distal axons reaches steady state within approximately 2 hr (Hawrot, 1982) and levels of cell body-associated ^{125}I-NGF reach steady-state within approximately 6 hr of continuous incubation of distal axons (Claude et al., 1982). The radioactivity in cell bodies is mostly intact ^{125}I-NGF which is eventually degraded and the degradation products released into the medium bathing the cell bodies and proximal axons (Ure and Campenot, 1994). Total retrograde transport is represented by the combined radioactivity from the cell bodies/proximal axons and from the medium bathing them.

Relationship Between Slow Receptor Binding on Distal Axons and Retrograde Transport of NGF

We first examined the relationship between ^{125}I-NGF binding to slowly-dissociating (slow) receptors on distal axons and ^{125}I-NGF retrograde transport. We measured slow receptor binding as the amount of axon-associated ^{125}I-NGF which was stable to a 2°C chase but was subsequently released from axons during a 37°C chase. Distal axons were incubated with ^{125}I-NGF (10-20 ng/ml) at 37°C for 11-21 hr, sufficient for steady-state association to be reached. In each experiment some cultures also received 100-200-fold excess NGF for determining nonspecific values for ^{125}I-NGF association and dissociation. The ^{125}I-NGF-containing medium in axon compartments in all cultures was then replaced with chase medium containing 1 μg/ml unlabeled NGF. Axons were chased at 2°C for 6 hours to dissociate ^{125}I-NGF from fast receptors and then subsequently chased with fresh chase medium for an additional 18 hr at 37°C to dissociate the ^{125}I-NGF from slow receptors. The amount of ^{125}I-NGF released from the slow receptors was compared to the amount of ^{125}I-NGF associated with axons at the beginning of the chase (determined in separate cultures) to estimate the proportion of axonal ^{125}I-NGF which was bound to slow receptors at steady-state. For these calculations only specific values for ^{125}I-NGF

association and dissociation were used (i.e. nonspecific values subtracted; see Experimental Procedures). Previous experiments suggested that ^{125}I-NGF released at 37°C represents dissociation from surface receptors rather than retroendocytosis from an intracellular pool and that approximately 85% of axonal ^{125}I-NGF was surface-bound (Ure and Campenot, 1996). Five experiments were performed, and their results are shown in Figure 5.1a. Slow receptor binding was found to represent 22-63% of the specific ^{125}I-NGF association to distal axons. The higher levels (41%, 63%) occurred in 16-day-old cultures, while the lower levels (22-33%) occurred in 37-42 day-old cultures. The combined ^{125}I-NGF dissociation from the 2 chase intervals averaged 83 ± 9% of the axonal ^{125}I-NGF, and the released radioactivity migrated on SDS-polyacrylamide gels as only intact ^{125}I-NGF (not shown). These results indicate that a considerable fraction of axonal ^{125}I-NGF was bound to slowly-dissociating receptors.

Retrograde transport rates were measured in sister cultures to those used for analysis of slow receptor binding to determine if the amount of slowly-dissociating ^{125}I-NGF correlated with the amount of ^{125}I-NGF which was retrogradely transported. ^{125}I-NGF concentrations were identical to those in the experiments above. Retrograde transport rates were calculated by dividing the total retrograde transport from the overnight ^{125}I-NGF incubation by the total transport period (15-20 hr), and then expressing the rate relative to the amount of ^{125}I-NGF specifically associated with axons at steady-state (final units = %/hr). Retrograde transport rates for the 5 experiments are shown in Figure 5.1b. We found that the fraction of axonal ^{125}I-NGF transported per hour (1.4-6.3%) correlated strongly with the fraction of axonal ^{125}I-NGF bound to slow receptors (Fig. 5.1c). That is, neurons which had high levels ^{125}I-NGF binding to slow receptors also transported ^{125}I-NGF at a high rate, consistent with an important role for slow receptors in retrograde transport.

The relationship between levels of slow receptor binding and retrograde transport was further examined by including another treatment group in the above experiments. In this group ^{125}I-NGF was supplied along with 150-fold excess NT-4 to distal axons. We expected excess NT-4 to be effective at reducing slow receptor binding, and in turn possibly ^{125}I-NGF transport, because Weskamp and Reichardt (1991) reported that half of the slow receptor binding on PC12 cells is dependent on ^{125}I-NGF binding to p75. The high concentration of NT-4 should have blocked virtually all binding of ^{125}I-NGF to p75, but additionally, it might have blocked some of the ^{125}I-NGF binding to trkA (Berkemeier et al., 1991; Ip et al., 1992). Following the overnight

incubation with ^{125}I-NGF plus NT-4, cultures were chased according to the protocol above. We found in all 3 experiments (culture ages = 16, 37, and 38 days-old) that ^{125}I-NGF dissociated from axons only during the 37°C chase, indicating that ^{125}I-NGF was bound only to slow receptors. However, as shown in Figure 5.2, the dissociation in these cultures was only 54% (range = 44-68%) as abundant as in control cultures which had been incubated with ^{125}I-NGF alone. Retrograde transport was also reduced in these cultures, by similar levels as slow ^{125}I-NGF dissociation. In the presence of excess NT-4, ^{125}I-NGF transport occurred to the level of 51% (range = 48-52%) of the transport in control cultures. These results further demonstrate an important relationship between slow receptor binding and retrograde transport, likely indicating that ^{125}I-NGF was transported primarily if not totally by slow receptors.

Contributions of trkA and p75 Binding to ^{125}I-NGF Association with Distal Axons

In the next experiments ^{125}I-NGF binding to trkA or p75 in distal axons was blocked with antibodies or unlabeled BDNF, and the effects on ^{125}I-NGF association and retrograde transport were examined. Results on ^{125}I-NGF association are presented first. Distal axons were incubated for 16-24 hr with 3-10 ng/ml ^{125}I-NGF plus one of the following additional treatments: 5% RTA antiserum or 500 μg/ml RTA IgG, which blocks ^{125}I-NGF binding to trkA (Clary et al., 1994); 4-5% REX antiserum or ≥150-fold molar excess of BDNF, both of which block ^{125}I-NGF binding to p75 (Weskamp and Reichardt, 1991; Rodríguez-Tébar et al., 1990); 5% normal rabbit antiserum or 500 μg/ml normal rabbit IgG as a control, or; ≥150-fold molar excess NGF for determination of nonspecific binding. Following the incubation distal axons were harvested to determine levels of specific ^{125}I-NGF association. Figure 5.3 shows the results of all experiments where the effects of at least 2 of the anti-receptor treatments were directly compared. We found that the contributions of p75 and trkA changed as cultures aged. In the youngest cultures RTA, REX and excess BDNF each reduced specific ^{125}I-NGF association by approximately 50%. However, as cultures aged a smaller fraction of ^{125}I-NGF association was reduced by RTA, while a larger fraction appeared to be reduced by REX and excess BDNF. These results indicate that ^{125}I-NGF association became relatively more p75-dependent as the neurons aged in culture. It is also important to note that REX and excess BDNF were equally effective at reducing ^{125}I-NGF association, suggesting that both treatments equally blocked ^{125}I-NGF binding to p75.

The effects of RTA, REX, and excess BDNF on ^{125}I-NGF transport were examined next (treatments described in preceeding section). Following the 16-24 hr incubation with 3-10 ng/ml ^{125}I-NGF plus one of the additional treatments, the total amount of ^{125}I-NGF which had been delivered to the cell body/proximal axon compartment (medium + cell extract radioactivity) during the incubation was quantified. The results are presented in Figure 5.4 (same cultures as in Fig. 5.3). Unlike the effects of the treatments on ^{125}I-NGF association, which varied with culture age (16-42 days old), the effects on ^{125}I-NGF transport were found not to vary significantly with age. An important role for trkA in ^{125}I-NGF transport was clearly evident, since RTA antibody (5% antiserum or 500 μg/ml IgG fraction) reduced specific transport by 80% on average.

The role of p75, however, was not clear, because interestingly, REX (4-5% antiserum) and excess BDNF (≥ 150-fold) reduced ^{125}I-NGF transport by varying degrees. REX reduced transport by 60% on average, whereas excess BDNF reduced transport by 25% on average. Although the results with excess BDNF were more variable than those with REX, ^{125}I-NGF transport in the presence of excess BDNF always exceeded transport in the presence of REX by a minimum of 33%. Because REX and excess BDNF similarly reduced ^{125}I-NGF association to distal axons (Fig. 5.3), the different effects of REX and BDNF on transport did not appear to arise because of varying abilities to block ^{125}I-NGF binding to p75. The higher level of transport in the presence of BDNF also did not appear to arise from the absence of pre-incubation with BDNF, as we found in one experiment that ^{125}I-NGF transport was the same, with or without an additional 5-hr BDNF treatment prior to incubation with ^{125}I-NGF plus excess BDNF (not shown).

Our results provide several suggestions about the role of p75 in ^{125}I-NGF transport. Firstly, they suggest that p75 contributed to at least 25% of ^{125}I-NGF transport, as implied by the effect of excess BDNF, although the contribution may have been as high as 60% of the transport, as implied by the effect of REX and possibly by the effects of excess NT-4 (previous section). Further experiments are required to resolve the exact contribution of p75. Proposed experiments include the following: (1) Compare the effects of REX IgG and REX Fab's on ^{125}I-NGF

transport to determine whether p75 crosslinking by REX IgG has an effect on ^{125}I-NGF transport; (2) Investigate the effects of anti-p75 peptides which block NGF binding to p75; (3) Examine the retrograde transport of mutant forms of NGF which binding only to trkA (Ibáñez et al., 1992) or only to p75 (Woo et al., 1995). The second suggestion from our results, based on the finding that approximately 20% of ^{125}I-NGF transport was not blocked by RTA, is that p75 partially functions as a carrier of ^{125}I-NGF to cell bodies, because ^{125}I-NGF should have been bound only to p75 in the presence of RTA. Finally, the different effects of REX and BDNF on ^{125}I-NGF transport but not on ^{125}I-NGF association suggest that the interaction of one or both of these molecules with p75 influenced ^{125}I-NGF transport by some mechanism. Since ^{125}I-NGF should have bound only to trkA when axons were incubated with REX or BDNF, it appears that under one or both of these experimental conditions that p75 regulated trkA-^{125}I-NGF transport.

^{125}I-BDNF and ^{125}I-NT-3 Retrograde Transport

The suggestion that p75 functioned partly as a neurotrophin carrier in retrograde transport was further explored by investigating whether ^{125}I-BDNF and ^{125}I-NT-3, which also bind p75, would also be retrogradely transported. Distal axons were incubated with medium containing ^{125}I-BDNF or ^{125}I-NT-3 (5-10 ng/ml), but without added NGF. As shown in Figure 5.5 (results from 1 of 3 experiments for each of ^{125}I-BDNF and ^{125}I-NT-3), virtually no ^{125}I-BDNF was transported, indicating that p75 was not significantly involved in ^{125}I-BDNF delivery to cell bodies. ^{125}I-NT-3 was transported to 9% of the level of ^{125}I-NGF, but since some ^{125}I-NT-3 transport likely occurred through trkA (Dechant et al., 1993) and possibly through trkC (Wetmore and Olson, 1995), the contribution of p75 was likely less than 9%. These results do not indicate a significant role for p75 in delivering BDNF or NT-3 to cell bodies, at least at the concentrations used in these experiments, and contrast with the suggested role in ^{125}I-NGF transport.

^{125}I-BDNF Association With Distal Axons

To confirm that ^{125}I-BDNF did associate with distal axons, ^{125}I-BDNF (5-10 ng/ml) was provided to distal axons for 16-22 hr, either alone or with 150-fold excess BDNF or NGF. The steady-state association was then measured. Four experiments were performed, using 15-35 day-old cultures, and the results from one of these experiments is shown in Figure 5.6 (35-day-old cultures). When ^{125}I-BDNF was supplied alone a measureable quantity of ^{125}I-BDNF did associate with axons, as compared to the background binding of ^{125}I-BDNF in side compartments whose axons were completely removed with distilled water prior to ^{125}I-BDNF incubation. The ^{125}I-BDNF association with axons was similar in quantity to the total ^{125}I-NGF association that occurred in the presence of RTA antibody (not shown), which was expected if ^{125}I-BDNF bound primarily to p75. Surprisingly, we were unable to determine the "specific" level of ^{125}I-BDNF association because a 150-fold excess of unlabeled BDNF, expected to compete ^{125}I-BDNF binding, actually increased ^{125}I-BDNF association with axons by nearly 2-fold, after accounting for the background binding to the culture dish. The augmented association was due to binding to the axons, since excess BDNF did not change background binding to the culture dish. A 150-fold excess of NGF, also expected to compete ^{125}I-BDNF binding to p75, did not change ^{125}I-BDNF association in the older cultures, indicating a similar effect to excess BDNF but to a lesser degree. A similar, 2-fold induction by excess BDNF was observed in 32 day-old cultures, while in 15- and 21-day-old cultures excess BDNF did not alter the level of total ^{125}I-BDNF association, indicating that the induction of ^{125}I-BDNF association was not as prominent in the younger cultures (not shown). Our results might be partially explained by positively cooperative binding of BDNF to p75, but also possibly by BDNF association with a high frequency, very low affinity binding site. At a minimum the results indicate that distal axons had a remarkable capacity for binding and/or sequestering BDNF.

DISCUSSION

Slowly-Dissociating Receptors and NGF Transport

The proportion of axonal ^{125}I-NGF which was bound to slow (slowly-dissociating) receptors correlated strongly with the proportion which was retrogradely transported per hour (transport rate). The levels of slow receptor binding which were measured, 20-60% of axonal ^{125}I-NGF, probably represented a minimum estimate of the true levels, since some of the ^{125}I-NGF bound to these receptors was likely transported during the 37°C chase in the assays to determine the level of binding. However, underestimating slow receptor binding should not have significantly altered the correlation with retrograde transport because the true, steady-state binding can be reasonably assumed to have been underestimated by a similar percentage in all experiments (i.e. similar ratio of the rate constants for dissociation:transport). A correlation between slow receptor binding and retrograde transport was also observed when ^{125}I-NGF binding to axons was blocked with an excess of NT-4. These results suggest that the receptors of most functional significance for retrograde transport were slowly-dissociating receptors.

High affinity receptors are reported to be important for NGF transport by sympathetic neurons in adult rats (Dumas et al., 1979) and for NGF internalization by PC12 cells (Bernd and Greene, 1984; Hosang and Shooter, 1987). Our results showing the importance of slow receptor binding are consistent with these previous results, insofar as high affinity receptors have been considered to be the same as slow receptors in the literature (Sutter et al., 1979; Landreth and Shooter, 1980; Schecter and Bothwell, 1981; Woodruff and Neet, 1986; Weskamp and Reichardt, 1991; Clary et al., 1994). Indeed, receptors must be slowly-dissociating if they are to be high affinity receptors. However, slow receptors do not necessarily have to bind NGF with high affinity but can also bind NGF with lower affinities (e.g. $K_D \approx 10^{-9}$ M), as exemplified by binding to PC12 cells (Schecter and Bothwell, 1981; Bernd and Greene, 1984; Woodruff and Neet, 1986) or to cells expressing trkA alone (Kaplan et al., 1991; Klein et al., 1991; Jing et al., 1992; Meakin et al., 1992). The slow receptors involved in NGF transport which we have investigated might indeed represent 2 or more distinct types of receptor, each with a different binding affinity. In a previous study we found that transport rates did not consistently vary to a

large extent across the NGF concentration range of 8 pM - 1.5 nM. Also we found in a direct comparison of transport rates at 0.15 nM and 1.5 nM NGF that the rates were only approximately 10% lower at 1.5 nM (Ure and Campenot, 1996). These observations are inconsistent with transport occurring only through high affinity receptors ($K_D \approx 10^{-11}$ M), considering that high affinity receptors typically represent less than 10% of all receptors on neurons (Meakin and Shooter, 1992). In vivo, Dumas et al. (1979) similarly found a relatively high transport rate at high NGF concentrations, which they interpreted as transport occurring through a class of high capacity-lower affinity receptors.

TrkA was likely a component of most if not all slow receptors in the neurons, since NGF dissociation from cells expressing only trkA occurs only with slow kinetics (Jing et al., 1992; Meakin et al., 1992; Mahadeo et al., 1994). Consistent with these findings, when NGF was permitted to bind only trkA in our experiments (i.e. coincubation with excess NT-4) or in previous experiments (Weskamp and Reichardt, 1991), NGF was observed to dissociate only with slow kinetics. However, some of the slow receptors also depended on ^{125}I-NGF binding to p75. In our experiments, when NGF binding to p75 was blocked with NT-4, slow dissociation was reduced by nearly one-half of control levels. Clary et al. (1994) similarly found in PC12 cells that REX reduced slow dissociation by approximately one-half. There are several possible models to explain how p75 might have participated in slow receptor formation. One model which has received much attention is the formation of a heteromeric complex between trkA and p75 in which the receptors share the binding of NGF dimers. A problem with this model is that there has been little success in being able to chemically crosslink trkA, p75, and NGF in a single complex (Barker and Murphy, 1992). Another potential model is that trkA might induce a slowly-dissociating form of p75 because of a physical interaction between trkA and p75. trkA might exert this effect by stabilizing p75 dimers through a physical interaction of extracellular domains (Wolf et al., 1995; Ross et al., 1996). Potentially, trkA might also stabilize p75-NGF interaction through intracellular signaling. One final possibility is that p75 might be able to independently form a slow receptor without interacting with trkA. Although p75 has been extensively shown to be a rapidly-dissociating receptor, there is also considerable evidence that it can behave as a slowly-dissociating, higher affinity receptor. Buxser observed that p75 receptors mixed with lipid vesicles behaved as slow receptors, and this form of p75 correlated with the appearance of high molecular weight p75 complexes (interpreted as dimers) in crosslinking assays. High molecular

weight complexes of p75 have in fact been a common observation in many studies (Grob et al., 1985; Green and Greene, 1986; Taniuchi et al., 1986a, b; Barker and Murphy, 1992). Green and Greene (1986) found in PC12 cells that a 103 kDa receptor-NGF complex identified by chemical crosslinking (likely representing p75-NGF) represented not only a low affinity/fast receptor complex but also a high affinity/slow receptor complex. Finally, p75 has frequently been reported to bind neurotrophins with positive cooperativity, which necessarily implicates p75 in binding with different affinities (Venkatakrishnan et al., 1990; Rodriguez-Tébar et al., 1990, 1992; Bothwell, 1995). According to Bothwell (1995), the affinities of neurotrophin binding to p75 can approach affinities typically regarded as high affinity binding for neurotrophins. Thus, we speculate that a fraction of the axonal p75 forms dimers which behave as slow NGF receptors and which participate in NGF transport.

Slow NGF receptors are insoluble in Triton X-100, likely reflecting an interaction with the cytoskeleton (Schecter and Bothwell, 1981; Vale and Shooter, 1982; Grob and Bothwell, 1983). It is tempting to speculate, therefore, that a cytoskeletal linkage contributes in some way to stabilizing the receptor complex. Also, the cytoskeletal linkage might enhance receptor signaling and retrograde transport, given the importance of slow receptors to these events. Similar hypotheses have been raised for other receptors which interact with the cytoskeleton (Pfeffer et al., 1987; Mao et al., 1992; Caplan et al., 1995; Gronowski and Bertics, 1995; Valitutti et al., 1995). For example, the EGF receptor binds EGF with 2 affinities even though there is no heterogeneity in primary receptor structure and even though each EGF molecule binds to only one receptor molecule. However, a distinct feature of the higher affinity receptor, which transmits the biological effects, is an association with the actin cytoskeleton (Van Bergen en Henegouwen et al., 1989; Den Hartigh et al., 1992). It can be predicted that all trkA but only a fraction of the p75 would be cytoskeletally-linked, reflecting their roles as slow receptors. Consistent with this prediction, trkA is reported to be clustered along axons, whereas p75 is distributed more diffusely (Kohn et al., 1996). Also, in fluorescence recovery experiments p75 is immobilized more in NGF-responsive cells (expressing slow receptors) than in nonresponsive cells (Venkatakrishnan et al., 1991), consistent with p75 contributing to some of the slow receptor binding. Cytoskeletal linkage might serve a role in retrograde transport by increasing the efficiency with which receptor-ladened vesicles associate with the transport mechanism. Sites where receptors are

cytoskeletally-linked might also be sites where molecules necessary for receptor activation and internalization are localized.

Age-Dependent Effects of Anti-Receptor Treatments on ^{125}I-NGF Association But Not on ^{125}I-NGF Transport

Reductions in ^{125}I-NGF association with distal axons resulting from anti-trkA (RTA) and anti-p75 (REX, excess BDNF) treatments were observed to be age-dependent. In younger cultures ^{125}I-NGF was reduced to a similar extent by both types of treatments, whereas in older cultures ^{125}I-NGF assocation was reduced more by anti-p75 treatments than by RTA. These findings suggest that the p75:trkA ratio increased with culture age, which might have resulted from the use of NGF at a high concentration (100 ng/ml) in the routine maintenance of the cultures. Miller et al. (1994) found that injection of exogenous NGF into the anterior chamber of the eye evoked an increase in the p75:trkA ratio, in terms of both the mRNA levels and in receptor immunostaining along the innervating, sympathetic axons. Also, we have previously observed continuously increasing levels of p75 mRNA in cultured neurons maintained in high concentrations of NGF (Ma et al., 1992).

In contrast to the effects of anti-receptor treatments on ^{125}I-NGF association, the relative effects of these treatments on ^{125}I-NGF transport did not vary with culture age. This would suggest that only a subset of the axonal receptors participated in ^{125}I-NGF transport and that the composition of those transport-efficient receptors did not change as cultures aged. As already discussed, slow receptors are interpreted to be the transport-efficient receptors, to which trkA and p75 both contribute. A principal role for trkA in ^{125}I-NGF transport is obvious from experiments using RTA antibody, but is also supported by the observation that as the contribution of trkA to ^{125}I-NGF association declined with age, so too was there a decline in the fraction of axonal ^{125}I-NGF transported per hour (data not shown, but see Ure and Campenot, 1996).

trkA Function in ^{125}I-NGF Transport

Distal axon treatment with RTA antibody reduced ^{125}I-NGF transport by approximately 80%, clearly demonstrating the importance of trkA binding for NGF delivery to cell bodies.

There are two general ways in which trkA might have functioned in retrograde transport, and these two scenarios also apply to how p75 might have functioned (discussed in the following section). Firstly, trkA might have functioned as a carrier of NGF to cell bodies. This likely was a prominent role, considering that tyrosine kinase receptors commonly internalize ligand (Sorkin and Waters, 1993). Ehlers et. al. (1995) demonstrated that retrogradely transported trkA in sciatic nerve was phosphorylated only when NGF was available at axon terminals, which could be interpreted as the transport of trkA-NGF complexes.

Secondly, however, trkA in its bound state might have functioned as a regulator of the transport of p75-NGF complexes. This could potentially result from a physical association between trkA and p75. Through this interaction, p75-NGF complexes might have "tagged onto" activated trkA as it was internalized. If trkA functioned partly in this way, then in our experiments the 80% of transport blocked by RTA might have represented an understimate of the trkA role because bivalent RTA crosslinks and activates trkA (Clary et al., 1996). Another possible way in which trkA might have regulated p75-NGF transport is through intracellular signaling. Perhaps p75 could transport NGF only if it was a downstream target of trkA signaling. In support of this possibility, a correlation exists between p75 involvement in internalization/transport and the effect of K252a, a blocker of trkA-mediated tyrosine phosphorylation. Kahle et al. (1994) reported that in PC12 cells p75 is not involved in NGF internalization, nor does K252a affect internalization. In contrast, we have observed that p75 does contribute to retrograde transport and that K252a does reduce retrograde transport by approximately one-half (not shown), which could suggest that p75 participation in NGF transport is dependent on trkA signaling.

p75 Function in ^{125}I-NGF Transport

REX and a large excess of BDNF are both reported to be effective at blocking NGF binding to p75, and we found that both reduced ^{125}I-NGF association by the same degree. In contrast we found that REX reduced ^{125}I-NGF transport by approximately 60% of control levels, but excess BDNF reduced transport by only 25% on average. These results suggest at a minimum that p75 was involved in NGF transport, but it is not clear whether the extent of involvement exceeded 25% of transport. Excess NT-4, which also competes ^{125}I-NGF binding to

p75, reduced transport by approximately 50%. While this would seem to support a larger contribution of p75, it is uncertain to what extent the reduction by NT-4 resulted from blocking p75 because NT-4 might also have competed binding to trkA (Berkemeier et al., 1991; Ip et al., 1992). The finding that ^{125}I-NGF transport still occurred in the presence of RTA, to the level of 20% of control transport, also is consistent with a role for p75. Moreover, the 20% involvement appears to have represented a carrier function for p75 because in the presence of RTA ^{125}I-NGF should have bound and have been delivered to cell bodies only by p75.

Given that p75 appeared to deliver some of the NGF to cell bodies, it was somewhat surprising that ^{125}I-BDNF was not retrogradely transported. One explanation for the lack of transport might be that NGF and BDNF interact with p75 differently, which is consistent with observations of the p75-mediated induction of the early gene, NFκB, in response to NGF binding but not BDNF binding (Carter et al., 1996). However, it is also possible that transport of NGF-p75 complexes might depend on trkA signaling. p75 might be capable of transporting neurotrophins only if trkA is activated. In the ^{125}I-BDNF experiments NGF was absent from the medium so trkA should not have been activated to a significant extent, whereas in ^{125}I-NGF experiments using RTA, trkA should have been activated because RTA crosslinks the receptor and elicits it biological effects (Clary et al., 1994). Investigating ^{125}I-BDNF transport under conditions where trkA is activated (e.g. when RTA or small quantities of NGF are supplied) would help to determine the role of trkA activation in p75 transport.

Two possibilities are considered below to reconcile the different effects of REX and excess BDNF on ^{125}I-NGF transport: a) firstly, that the real level of p75 involvement was 25% of ^{125}I-NGF transport, as demonstrated by BDNF treatment, and that REX artifactually inhibited transport to an even greater extent due to its binding to p75 (e.g. possibly p75 crosslinking), or; b) secondly, that the real level of p75 involvement transport was 60% of ^{125}I-NGF transport, as demonstrated by REX treatment, but that BDNF did not reduce transport by this amount because BDNF binding to p75 augmented ^{125}I-NGF transport. A combination of these explanations is also possible. In either (a) or (b), it is significant to note that p75-REX and/or p75-BDNF complexes are necessarily implicated in regulating the retrograde transport of trkA-^{125}I-NGF, since ^{125}I-NGF should have associated only with trkA and be carried to cell bodies only by trkA during REX or excess BDNF incubation. The effects of REX and BDNF require further investigation to be resolved, but we hypothesize that BDNF gave the false indication of the contribution of p75. We

propose that NGF binding to p75 normally enhances retrograde transport of trkA-NGF complexes through a regulatory mechanism and that in our experiments BDNF binding to p75 mimicked this positive, regulatory effect. Furthermore, a prediction can be made that any neurotrophin could facilitate transport of trk-neurotrophin complexes, since neurotrophins ubiquitously bind p75. In support of this possibility, Curtis et al. (1995) found that small doses of NGF injected into targets of sensory neurons in vivo augmented retrograde transport of coinjected ^{125}I-BDNF and ^{125}I-NT-4. Another observation which is consistent with this hypothesis is that the MC192 antibody to p75, which increases p75 affinity for NGF (Chandler et al., 1984), increases NGF transport (Taniuchi and Johnson, 1985; also unpublished observations).

To explain how a p75-neurotrophin interaction might augment trkA binding and in turn retrograde transport, Bothwell (1995) proposed a model in which high affinity, p75 dimers resulting from positively cooperative binding (Venkatakrishnan et al., 1990; Rodriguez-Tébar et al., 1990, 1992; Bothwell, 1995) interact with trkA in some manner to increase trkA affinity and in turn increase the association of NGF with trkA. Neurotrophins that bound p75 with high cooperativity (e.g. BDNF) were predicted to be most effective at increasing trkA binding and in turn augment the responses resulting from trkA binding. Our data suggesting that BDNF augmented trkA-^{125}I-NGF transport is consistent with this allosteric model. Also consistent with this model is a correlation between the magnitude of cooperative binding by various neurotrophins and the reported extent of involvement of p75 in retrograde transport of these neurotrophins in sensory neurons (BDNF $\rangle$ NT-3 $\rangle$ NGF; Bothwell, 1995; Curtis et al., 1995). High affinity, p75 dimers might facilitate trkA binding by stabilizing trkA dimers, perhaps through association of extracellular domains (Wolf et al., 1995; Ross et al., 1996). Alternatively, they might be capable of regulating trkA endocytosis by means of the mastoparan-like domain of p75, since mastoparan has been reported to regulate endocytosis through its effects on G-proteins (Colombo et al., 1992; Carter et al., 1993; Eker et al., 1994).

A different model of p75-trkA interaction which has received some support (Barker and Shooter, 1994) is the presentation model, but our data are less consistent with this model. According to this model, p75 augments trkA binding by locally concentrating NGF in the vicinity of trkA. If p75 functioned solely by this mechanism, then in our experiments REX and excess BDNF, which appeared to equally block ^{125}I-NGF binding to p75, should not have affected

transport differently. By the same argument, our data are also not consistent with p75 and trkA forming heterodimers and sharing the binding of individual NGF dimers.

Our results suggesting a role for p75 in ^{125}I-NGF transport are consistent with previous findings that p75 is retrogradely transported in sympathetic neurons and that MC192 antibody augments ^{125}I-NGF transport. In contrast, Curtis et al. (1995) found that blocking ^{125}I-NGF binding to p75 with REX or soluble, truncated, p75 receptor did not have a statistically significant effect on ^{125}I-NGF transport by sympathetic neurons in vivo, although a trend towards reduced transport is discernible from the data. In sensory neurons they found p75 did contribute to approximately 30% of ^{125}I-NGF transport. No satisfactory explanation can be offered at this time for the different observations between their study and the present study on the p75 role in sympathetic neurons, particularly since REX was used in both studies. However, the experimental techniques in the 2 studies are markedly different, and also it is possible that a real difference in the role of p75 does exist between young neurons growing in culture as compared to mature neurons in adult animals.

^{125}I-BDNF Association With Distal Axons

A 150-fold excess of BDNF failed to compete ^{125}I-BDNF association with axons. On the contrary, the association was increased nearly 2-fold in cultures older than 30 days. These results demonstrate a tremendous capacity of distal axons for BDNF association, which very likely involved p75 because no other BDNF receptors are known to exist in sympathetic neurons. The most likely explanation for an *increase* in ^{125}I-BDNF association is that BDNF bound p75 with strong positive cooperativity. That is, the affinity of p75 markedly increased when excess BDNF was added. Positively cooperative binding to p75 has previously been reported for BDNF, NT-3, and to a small extent for NGF (Venkatakrishnan et al., 1990; Rodriguez-Tébar et al., 1990, 1992; Bothwell, 1995). Positive cooperativity commonly reflects changes in quarternary structure in oligomeric proteins (Perutz, 1990), although in the case of lamprey hemoglobin a complete dissociation of subunits is believed to be responsible for increasing hemoglobin affinity for oxygen (Dohi et al., 1973; Perutz, 1990; Nikinmaa et al., 1995). Thus, a reasonable hypothesis for p75 is that it exists as dimers in both the neurotrophin-bound and unbound states but that

subtle structural changes occur in the dimers upon neurotrophin binding to permit a fraction of the dimers to adopt a high affinity conformation.

Cooperative binding could also explain the *lack* of an effect of excess BDNF on ^{125}I-BDNF association which we observed in cultures younger than 30 days old, although an alternative explanation might be that p75 was extremely abundant on axons and bound BDNF with an affinity much lower than ≈ 1 nM as commonly reported (Radeke et al., 1987; Clary et al., 1994; Mahadeo et al., 1994). A very low affinity p75 (e.g. $K_D \approx 10^{-7}$ M) could permit binding of high concentrations of BDNF used in our experiments (60 nM) without significantly competing ^{125}I-BDNF binding. Some studies have, in fact, suggested a K_D in excess of 10 nM (Taniuchi et al., 1986a; Vissavajjhala and Ross, 1990). As yet unknown BDNF binding sites besides p75 that are of high frequency and low affinity might also have contributed to the BDNF association.

If a very low affinity form of p75 was responsible for the excessive BDNF binding, then it could reasonably be predicted that ^{125}I-NGF, which binds with a similar steady-state affinity to p75 (Rodríguez-Tébar et al., 1990), also would not be competed by excess NGF. Yet, we observed that ^{125}I-NGF association was always competed by excess NGF. On the other hand, BDNF and NGF binding to p75 might be different and this might explain the different association of ^{125}I-BDNF and ^{125}I-NGF. The positively charged residues in BDNF which are important for p75 binding, are uniquely distributed in the molecule as compared to their distribution in other neurotrophins (Rydén et al., 1995).

Several observations from our experiments support a small degree of cooperative NGF binding, which corroborates previous reports (Woodruff and Neet, 1986; Venkatakrishnan et al., 1990; Bothwell, 1995). Firstly, in the 42-day-old cultures shown in Figure 3 REX and excess BDNF reduced ^{125}I-NGF association to a greater extent than did excess NGF, suggesting that excess NGF augmented ^{125}I-NGF association above truly "nonspecific" levels. Secondly, dissociation assays in ≥ 37-day-old cultures suggest that more ^{125}I-NGF becomes bound to rapidly-dissociating receptors (i.e. p75) in the presence of excess NGF than in the presence of excess NT-4 (not shown). Thirdly, we have observed that a 10-fold range in the concentration of ^{125}I-NGF applied to distal axons produces a 12-fold range in ^{125}I-NGF association (not shown). Cooperative binding can complicate the determination of "specific" (higher affinity) binding because the true level of nonspecific binding (i.e. normally representing ^{125}I-ligand binding in the presence of excess, unlabeled ligand) is obscured. However, the conclusions from this study

surrounding ^{125}I-NGF association are unlikely to be significantly affected by cooperative NGF binding because the cooperativity appeared to occur to only a minor extent.

Conclusions

Important roles for slow receptors and trkA in ^{125}I-NGF transport have been demonstrated. The role of p75 remains enigmatic but it too contributed to ^{125}I-NGF transport, at least to a minor extent. Beyond the quantitative contributions of the receptors, this study has begun to address how trkA and p75 might function in transport. Although more experiments are required to resolve these functions, the present data raise the possibility that both receptors might function not only as NGF carriers but as regulators of each other's transport.

What might be the functional significance of p75, given that it internalizes neurotrophins inefficiently, that its binding occurs with positive cooperativity, and that it affects trkA binding (Barker and Shooter, 1994), trkA signaling (Barker and Shooter, 1994; Kahle et al., 1994; Verdi et al., 1994), and retrograde transport? These characteristics would seem to make p75 an ideal sensor of trophic environments. The advantage of positive cooperativity for a receptor is that small changes in free ligand would translate into relatively large changes in receptor occupancy. Changes in p75 occupancy could then be conveyed to trkA, resulting in responses (including retrograde transport) appropriate to the surrounding concentration of trophic factor. In essence, axonal p75 might serve as a "positive-gain" receptor. The low internalization rate can be predicted to be beneficial by maintaining p75 in the axonal membrane where it is able to sample the extracellular environment.

REFERENCES

Barker, P. A., and Murphy, R. A. (1992). The nerve growth factor receptor: a multicomponent system that mediates the actions of the neurotrophin family of proteins. Mol. Cell. Biochem. *110*, 1-15.

Barker, P. A., and Shooter, E. M. (1994). Disruption of NGF binding to the low affinity neurotrophin receptor $p75^{LNTR}$ reduces NGF binding to trkA on PC12 cells. Neuron *13*, 203-215.

Bernd, P., and Grerene, L. A. (1984). Association of ^{125}I-nerve growth factor with PC12 pheochromocytoma cells. J. Biol. Chem. *259*, 15509-15516.

Berkemeier, L. R., Winslow, J, W., Kaplan, D. R., Kikolics, K., Goeddel, D. V., and Rosenthal, A. (1991). Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. Neuron *7*, 857-866.

Bothwell, M. (1995). Functional interactions of neurotrophins and neurotrophin receptors. Annu. Rev. Neurosci. *18*, 223-253.

Buxser, S., Puma, R., and Johnson, G. L. (1985). Properties of the nerve growth factor receptor. J. Biol. Chem. *260*, 1917-1926.

Caplan, S., Zeliger, S., Wang, L., and Baniyash, M. (1995). Cell-surface-expressed T-cell antigen-receptor ξ chain is associated with the cytoskeleton. Proc. Natl. Acad. Sci. USA *92*, 4768-4772.

Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhäuser, N., Böhm-Matthaei, R., Baeuerle, P. A., and Barde, Y-A. (1996). Selective activation of NF-κB by nerve growth factor through the neurotrophin receptor p75. Science *272*, 542-545.

Carter, L. L., Redelmeier, T. E., Woollenweber, L. A., and Schmid, S. L. (1993). Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. J. Cell Biol. *120*, 37-45.

Chandler, C. E., Parsons, L..M., Hosang, M., and Shooter, E. M. (1984). A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J. Biol. Chem. *259*, 6882-6889.

Chao, M. V. (1994). The p75 neurotrophin receptor. J. Neurobiol. *25*, 1373-1385.

Clary, D. O., Weskamp, G., Austin, L. R., and Reichardt, L. F. (1994). TrkA cross-linking mimics neuronal responses to nerve growth factor. Mol. Biol. Cell *5*, 549-563.

Claude, P., Hawrot, E., Dunis, D. A., Campenot, R. B. (1982). Binding, internalization, and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J. Neurosci. *2*, 431-442.

Colombo, M. I., Mayorga, L. S., Casey, P. J., and Stahl, P. D. (1992). Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. Science *255*, 1695-1697.

Curtis, R., Adryan, K. M., Stark, J. L., Park, J., Compton, D. L., Weskamp, G., Huber, L. J., Chao, M. V., Jaenisch, R., Lee, K.-F., Lindsay, R. M., and DiStefano, P. S. (1995). Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron *14*, 1201-1211

Dohi, Y., Sugita, Y., and Yoneyama, Y. (1973). The self-association and oxygen equilibrium of hemoglobin from the lamprey, *Entosphenus japonicus*. J. Biol. Chem. *248*, 2354-2363.

Dechant G, Rodriguez-Tébar A, Kolbeck R, Barde Y-A (1993) Specific high-affinity receptor for neurotrophin-3 on sympathetic neurons. J. Neurosci. 13:2610-2616.

Den Hartigh, J. C., Van Bergen en Henegouwen, P. M. P., Verkleij, A. J., and Boonstra, J. (1992). The EGF receptor is an actin-binding protein. J. Cell Biol. *119*, 349-355.

Dumas, M., Schwab, M. E., and Thoenen, H. (1979). Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. J. Neurobiol. *10*, 179-197.

Ehlers, M. D., Kaplan, D. R., Price, D. L., and Koliatsos, V. E. (1995). NGF-stimulated retrograde transport of trkA in the mammalian nervous system. J. Cell Biol. *130*, 149-156.

Eker, P., Holm, P. K., van Deurs, B., and Sandvig, K. (1994). Selective regulation of apical endocytosis in polarized Madin-Darby canine kidney cells by mastoparan and cAMP. J. Biol. Chem. *269*, 18607-18615.

Godfrey, E. W., and Shooter, E. M. (1986). Nerve growth factor receptors on chick embryo sympathetic ganglion cells: binding characteristics and development. J. Neurosci. *6*, 2543-2550.

Green, S. H., and Greene, L. A. (1986). A single $M_r \approx 103{,}000$ ^{125}I-β-nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. J. Biol. Chem. *261*, 15316-15326.

Grob, P. M., and Bothwell, M. A. (1983). Modification of nerve growth factor receptor properties by wheat germ agglutinin. J. Biol. Chem. *258*, 14136-14143.

Grob, P. M., Ross, A. H., Koprowski, H., and Bothwell, M. (1985). Characterization of the human melanoma nerve growth factor receptor. J. Biol. Chem. *260*, 8044-8049.

Gronowski, A. M., and Bertics P. J. (1995). Modulation of epidermal growth factor receptor interaction with the detergent-insoluble cytoskeleton and its effects on receptor tyrosine kinase activity. Endocrinol. *136*, 2198-2205.

Hawrot, E. (1982). Characteristics of the association of nerve growth factor with primary cultures of rat sympathetic neurons. J. Neurosci. Res. *8*, 213-224.

Hosang, M., and Shooter, E. M. (1987). The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. EMBO J. *6*, 1197-1202.

Ibáñez, C. F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T. L., and Persson, H. (1992). Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. Cell *69*, 329-341.

Ip, N. Y, Ibáñez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., Le Beau, M. M., Espinosa III, R., Squinto, S. P., Persson, H., and Yancopoulos, G. D. (1992). Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution, and receptor specificity. Proc. Natl. Acad. Sci. USA *89*, 3060-3064.

Jing, S., Tapley, P., and Barbacid, M. (1992). Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron *9*, 1067-1079.

Johnson, E. M. Jr., Taniuchi, M., Clark, H. B., Springer, J. E., Koh, S., Tayrien, M. W., and Loy, R. (1987). Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. J. Neurosci. *7*, 923-929.

Kahle, P., and Hertel, C. (1992). Nerve growth factor (NGF) receptor on rat glial cell lines. Evidence for NGF internalization via $p75^{NGFR}$. J. Biol. Chem. *267*, 13917-13923.

Kahle, P., Barker, P. A., Shooter, E. M., and Hertel, C. (1994). p75 nerve growth factor receptor modulates $p140^{trkA}$ kinase activity, but not ligand internalization, in PC12 cells. J. Neurosci. Res. *38*, 599-606.

Klein, R., Jing, S., Nanduri, V., O'Rourke, E., and Barbacid, M. (1991). The trk proto-oncogene encodes a receptor for nerve growth factor. Cell *65*, 189-197.

Landreth, G. E., and Shooter, E. M. (1980). Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low- to high-affinity states. Proc. Natl. Acad. Sci. USA *8*, 4751-4755.

Le Bivic, A., Sambuy, Y., Patzak, A., Patil, N., Chao, M., and Rodriguez-Boulan, E. (1991). An internal deletion in the cytoplasmic tail reverses the apical localization of human NGF receptor in transfected MDCK cells. J. Cell Biol. *115*, 607-618.

Loy, R., Lachyankar, M. B., Condon, P. J., Poluha, D. K., and Ross, A. H. (1994). Retrograde axonal transport and lesion-induced upregulation of the trkA high-affinity NGF receptor. Exp. Neurol. *130*, 377-386.

Lucidi-Phillipi, C. A., Clary, D. O., Reichardt, L. F., and Gage, F. H. (1996). TrkA activation is sufficient to rescue axotomized cholinergic neurons. Neuron *16*, 653-663.

Ma, Y., Campenot, R. B., and Miller, F. D. (1992). Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J. Cell Biol. *117*, 135-141.

Mahadeo, D., Kaplan, L., Chao, M. B., and Hempstead, B. L. (1994). High affinity nerve growth factor binding displays a faster rate of association than $p140^{trk}$ binding. J. Biol. Chem. *269*, 6884-6891.

Mao, S.-Y., Alber, G., Rivera, J., Kochan, J., and Metzger, H. (1992). Interaction of aggregated native and mutant IgE receptors with the cellular skeleton. Proc. Natl. Acad. Sci. USA *89*, 222-226.

Meakin, S. O, and Shooter, E. M. (1992). The nerve growth factor family of receptors. TINS *15*, 323-331.

Meakin, S. O., Suter, U., Drinkwater, C., Welcher, A. A., and Shooter, E. M. (1992). The rat *trk* protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc. Natl. Acad. Sci. USA *89*, 2374-2378.

Miller, F. D., Speelman, A., Mathew, T. C., Fabian, J., Chang, E., Pozniak, C., and Toma, J. G. (1994). Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. Dev. Biol. *161*, 206-217.

Nikinmaa, M., Airaksinen, S., and Virkki, L. V. (1995). Haemoglobin function in intact lamprey erythrocytes: Interactions with membrane function in the regulation of gas transport and acid-base balance. J. Exp. Biol. *198*, 2423-2430.

Olender, E. J., and Stach, R. W. (1980). Sequestration of ^{125}I-labeled β nerve growth factor by sympathetic neurons. J. Biol. Chem. *255*, 9338-9343.

Olender, E. J., Wagner, JB. J., and Stach, R. W. (1981). Sequestration of ^{125}I-labeled β nerve growth factor by embryonic sensory neurons. J. Neurochem. *37*, 436-442.

Perutz, M. (1990). Mechanisms of Cooperativity and Allosteric Regulation in Proteins. Cambridge, UK: Cambridge University Press.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., and Shooter, E. M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature *325*, 593-597.

Rodríguez-Tébar, A., Dechant, G., Gotz, R., and Barde, Y. A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron *4*, 487-492.

Rodriguez-Tébar, A., Dechant, G., Gotz, R., and Barde, Y. A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J. *11*, 917-922.

Ross, A. H., Daou, M.-C., McKinnon, C. A., Condon, P. J., Lachyankar, M. B., Stephens, R. M., Kaplan, D. R., Wolf, D. E. (1996). The neurotrophin receptor, gp75, forms a complex with the receptor tyrosine kinase TrkA. J. Cell Biol. *132*, 945-953.

Rydén, M., Murray-Rust, J., Glass, D., Ilag, L. L., Trupp, M., Yancopoulos, G. D., McDonald, N. Q., and Ibáñez, C. F. (1995). Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for $p75^{LNGFR}$ in NT-4 signaling. EMBO J. *14*, 1979-1990.

Schechter, A. L., and Bothwell, M. A. (1981). Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. Cell *24*, 867-874.

Smeyne, R. J., Klein, R., Schnapp, A., Long, L. K., Bryant, S., Lewin, A., Lira, S. A., and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature *368*, 246-248.

Sorkin, A., and Waters, C. M. (1993). Endocytosis of growth factor receptors. Bioessays *15*, 375-382.

Stach, R. W., and Wagner, B. J. (1982). Sequestration requirements for the degradation of ^{125}I-labeled β nerve growth factor bound to embryonic sensory neurons. J. Neurosci. Res. *5*, 403-411.

Sutter, A., Riopelle, R. J., Harris-Warrick, R. M., and Shooter, E. M. (1979). Nerve growth factor. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J. Biol. Chem. *254*, 5972-5982.

Taniuchi, M., and Johnson, E. M. Jr. (1985). Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directed against the rat nerve growth factor receptor. J. Cell Biol. *101*, 1100-1106.

Taniuchi, M., Johnson, E. M. Jr., Roach, P. J., and Lawrence, J. C. Jr. (1986a). Phosphorylation of nerve growth factor receptor proteins in sympathetic neurons and PC12 cells. J. Biol. Chem. *261*, 13342-13349.

Taniuchi, M., Schweitzer, J. B., and Johnson, E. M. Jr. (1986b). Nerve growth factor receptor molecules in rat brain. Proc. Natl. Acad. Sci. USA *83*, 1950-1954.

Ure, D. R., and Campenot, R. B. (1996). Retrograde transport and steady-state distribution of ^{125}I-NGF in rat sympathetic neurons in compartmented cultures. J. Neurosci. *in press*.

Vale, R. D., and Shooter, E. M. (1982). Alteration of binding properties and cytoskeletal attachment of nerve growth factor receptors in PC12 cells by wheat germ agglutinin. J. Cell Biol. *94*, 710-717.

Valitutti, S., Dessing, M., Aktories, K., Gallati, H., and Lanzavecchia, A. (1995). Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. J. Exp. Med. *181*, 577-584.

Van Bergen en Henegouwen, P. M. P., Defize, L. H., K., De Kroon, J, Van Damme, H., Verkleij, A. J., and Boonstr, J. (1989). Ligand-induced association of epidermal growth factor receptor to the cytoskeleton of A431 cells. J. Cell. Biochem. *39*, 455-465.

Venkatakrishnan, G., McKinnon, C. A., Ross, A. H., and Wolf, D. E., (1990). Lateral diffusion of nerve growth factor receptor: modulaton by ligand-binding and cell-associated factors. Cell. Regul. *1*, 605-614.

Verdi, J. M., Birren, S. J., Ibánez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994). $p75^{LNGFR}$ regulates trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron *12*, 733-745.

Weskamp, G., and Reichardt, L. F. (1991). Evidence that biological activity of nerve growth factor is mediated through a novel subclass of high affinity receptors. Neuron *6*, 649-663.

Wetmore, C., and Olson, L. (1995). Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. J. Comp. Neurol. *353*, 143-159.

Wolf, D. E., McKinnon, C. A., Daou, M.-C., Stephens, R. M., Kaplan, D. R., and Ross, A. (1995). Interaction with trkA immobilize gp75 in the high affinity nerve growth factor receptor complex. J. Biol. Chem. *270*, 2133-2138.

Woo, S. B., Timm, D. E., and Neet, K. E. (1995). Alteration of NH_2-terminal residues of nerve growth factor affects activity and trk binding without affecting stability or conformation. J. Biol. Chem. *270*, 6278-6285.

Woodruff, N. R., and Neet, K. E. (1986). β Nerve growth factor binding to PC12 cells. Association kinetics and cooperative interactions. Biochem. *25*, 7956-7966.

FIGURES

Figure 5.1 Correlation between ^{125}I-NGF binding to slowly-dissociating receptors and ^{125}I-NGF retrograde transport rate

Distal axons were incubated at 37°C for 11-21 hr with 10-20 ng/ml ^{125}I-NGF, either alone or in combination with 125-200-fold excess NGF (for determination of nonspecific binding). Cultures were then split into 3 groups: i) Axons from some cultures were harvested to determine levels of axon-associated ^{125}I-NGF at steady-state (6-7 axon compartments/treatment/experiment); ii) In some cultures ^{125}I-NGF-containing medium was exchanged with medium containing 1 μg/ml NGF, and the axons chased for 6 hr at 2°C. Axon medium was then exchanged a second time, and the cultures further incubated for 18 hr at 37°C, during which ^{125}I-NGF bound to slowly-dissociating receptors was released into the bathing medium and collected (6-7 axon compartments/treatment/ experiment); iii) In some cultures the amount of retrogradely transported ^{125}I-NGF was determined by collecting all radioactivity from each cell body/proximal axon compartment (medium + cell extract; 3 cultures/treatment/experiment). (A) ^{125}I-NGF dissociated from slow receptors is expressed as a percentage of the axon-associated ^{125}I-NGF, with nonspecific values for dissociation and association subtracted. (B) Retrogradely transported ^{125}I-NGF is expressed as a percentage of the axon-associated ^{125}I-NGF, with nonspecific values for transport and association subtracted. (C) Data from (A) and (B) showing correlation between retrograde transport rate and proportion of axonal ^{125}I-NGF association which was to slow receptors.

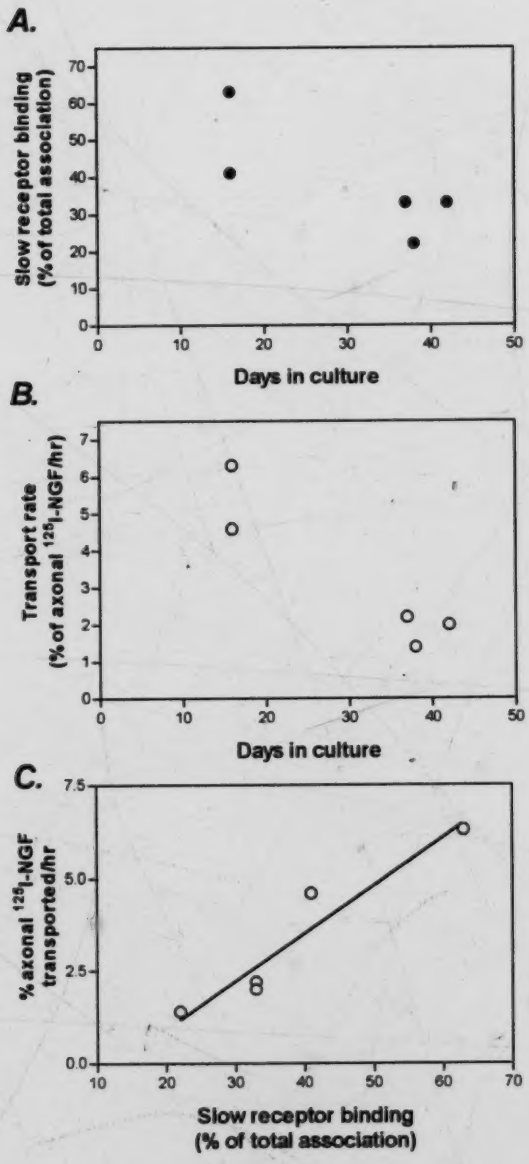

A.
Slow receptor binding
(% of total association)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
Days in culture
B.
Transport rate
(% of axonal 125I-NGF/hr)
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Days in culture
C.
% axonal 125I-NGF
transported/hr
7.5
5.0
2.5
0.0
10
20
30
40
50
60
70
Slow receptor binding
(% of total association)

Figure 5.2 Reductions in ^{125}I-NGF binding to slow receptors and in ^{125}I-NGF retrograde transport by excess NT-4

Distal axons were incubated at 37°C for 11-21 hr with 10-20 ng/ml ^{125}I-NGF, either alone or in combination with 150-fold excess NT-4 or 150-fold excess NGF (for determination of nonspecific binding). Cultures were then split into 2 groups: i) In some cultures the specific level of slowly- ^{125}I-NGF dissociation was measured as in Fig. 5.1 (6-7 axon compartments/ treatment/experiment); ii) In some cultures the specific level of retrogradely transported ^{125}I-NGF was quantified as in Fig. 5.1 (3 cultures/ treatment/ experiment). Shown are mean, relative levels (± SEM) of slow receptor binding and retrograde transport from 3 experiments, with nonspecific values subtracted.

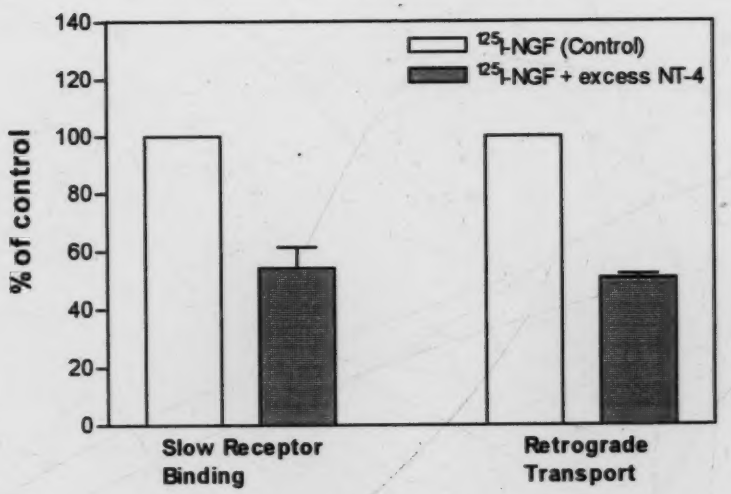

140
120
100
80
60
40
20
0
% of control
125I-NGF (Control)
125I-NGF + excess NT-4
Slow Receptor Binding
Retrograde Transport

Figure 5.3 Contributions of p75 and trkA to ^{125}I-NGF association with distal axons

Distal axons were incubated at 37°C for 15-24 hr with 5-10 ng/ml ^{125}I-NGF plus one of the following: 5% normal rabbit antiserum (control), 5% RTA antiserum (*circles*), 4-5% REX antiserum (*squares*), 150-200-fold excess BDNF (*triangles*), or 150-200-fold excess NGF (for determining nonspecific association). In each experiment 4-8 distal axon compartments were used per treatment. Distal axons were then harvested to quantify associated ^{125}I-NGF. Association is expressed relative to control levels (means ± SEM), with nonspecific association subtracted..

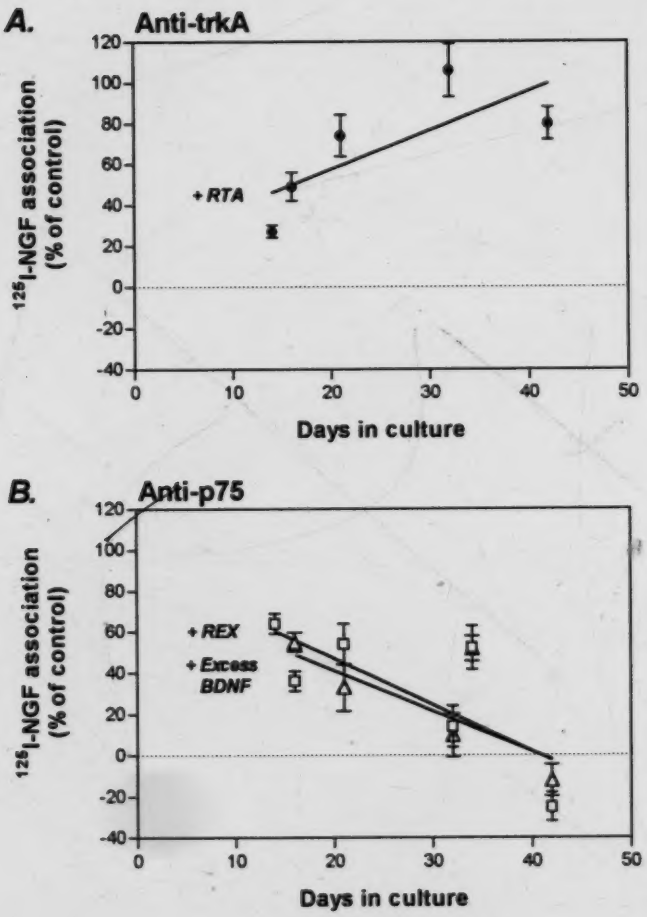

A.
Anti-trkA
^{125}I-NGF association (% of control)
+ RTA
Days in culture
B.
Anti-p75
^{125}I-NGF association (% of control)
+ REX
+ Excess BDNF
Days in culture

Figure 5.4 Contributions of trkA and p75 to ^{125}I-NGF retrograde transport

Retrograde transport was measured in the cultures described in Fig. 5.3. Following the distal axon incubation with ^{125}I-NGF plus one of the additional treatments, all radioactivity from each cell body/proximal axon compartment (medium + cell extract) was quantified (2-4 cultures/treatment/experiment). Transport is expressed relative to control transport (means ± SEM), with nonspecific transport subtracted.

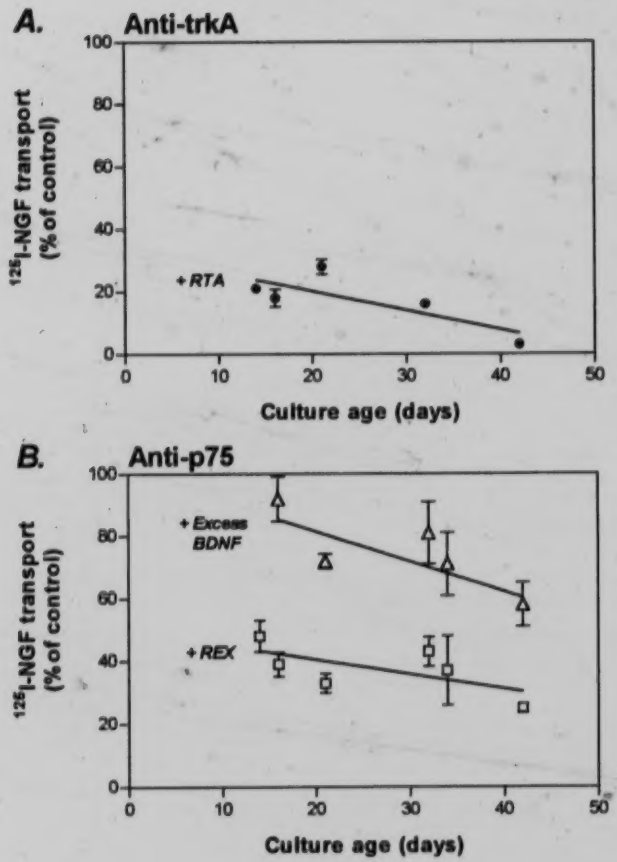

A.
Anti-trkA
^{125}I-NGF transport (% of control)
+RTA
Culture age (days)
B.
Anti-p75
^{125}I-NGF transport (% of control)
+ Excess BDNF
+REX
Culture age (days)

Figure 5.5 Retrograde transport of ^{125}I-BDNF and ^{125}I-NT-3

In 21-day-old (A) or 16-day-old (B) cultures, distal axons were incubated at 37°C with the indicated neurotrophins. Iodinated neurotrophins were supplied at 5 ng/ml (A) or 10 ng/ml (B), and unlabeled neurotrophins were provided in 200-fold excess of the labeled neurotrophins. Incubation periods were: (A) 18.5 hr for ^{125}I-NGF, 22 hr for ^{125}I-BDNF; (B) 27 hr for both ^{125}I-NGF and ^{125}I-NT-3. Total accumulations of radioacitivity in cell body/proximal axon compartments were then measured. Shown are levels of retrograde transport, expressed as means and ranges from 1-3 cultures/treatment.

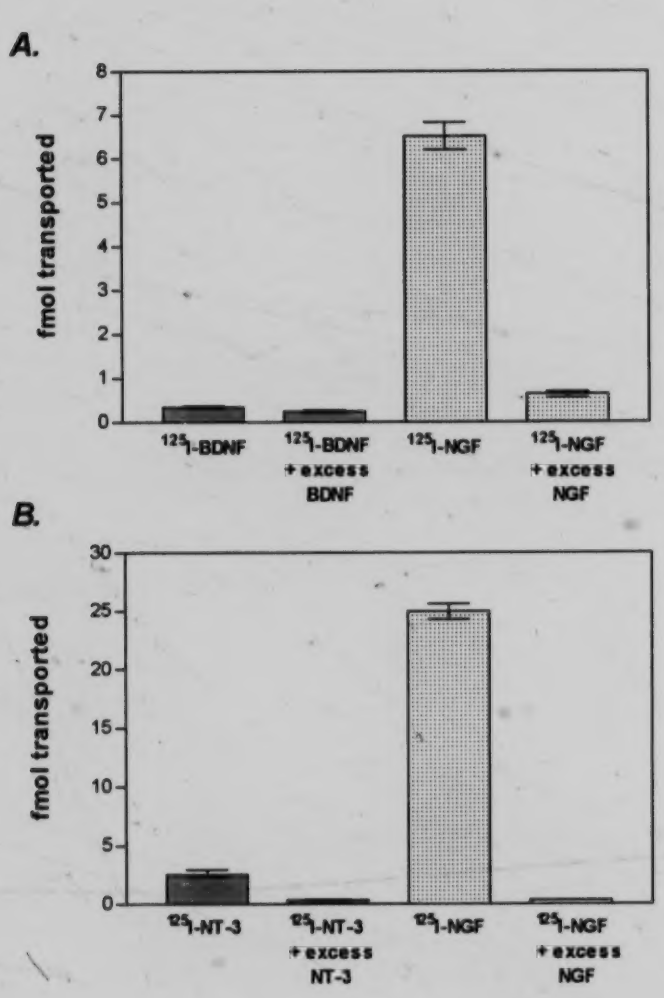
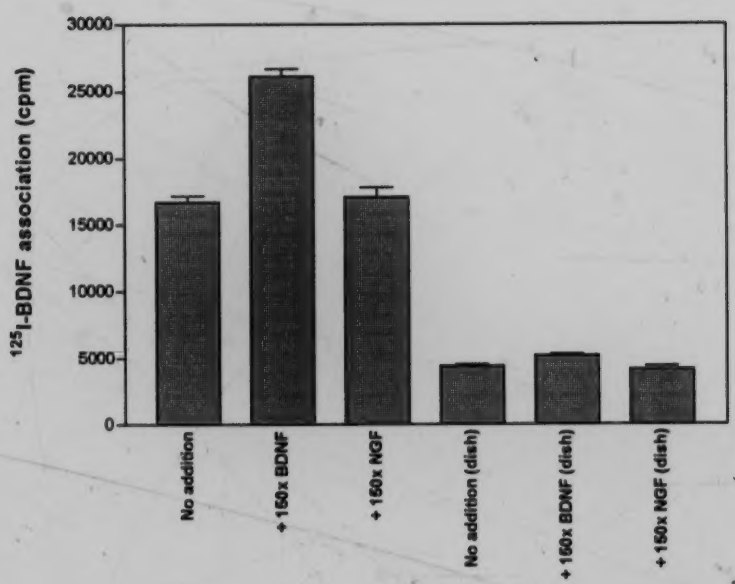

A.
fmol transported
8
7
6
5
4
3
2
1
0
125I-BDNF
125I-BDNF + excess BDNF
125I-NGF
125I-NGF + excess NGF
B.
fmol transported
30
25
20
15
10
5
0
125I-NT-3
125I-NT-3 + excess NT-3
125I-NGF
125I-NGF + excess NGF

Figure 5.6 ^{125}I-BDNF association with distal axons

In intact 35-day-old cultures and in cultures in which distal axons were removed with distilled water prior to the experiment 10 ng/ml ^{125}I-BDNF was supplied to the side (distal axon) compartments, either alone or in combination with 150-fold excess BDNF or NGF. After an 18 hr incubation at 37°C the ^{125}I-BDNF associated with distal axons and/or with the dish was measured. Each bar represents the mean level of radioactivity ± SEM from 6 axon compartments.


^{125}I-BDNF association (cpm)
30000
25000
20000
15000
10000
5000
0
No addition
+ 150x BDNF
+ 150x NGF
No addition (dish)
+ 150x BDNF (dish)
+ 150x NGF (dish)

Chapter 6

Summary and Conclusions

GENERAL COMMENTS

Studies With Cell Cultures

Key results from this thesis research are summarized below and discussed in the context of previous literature. One general point of discussion concerns the use of cell cultures as an experimental model. Caution should be taken in drawing parallels between observations from culture models, as used presently, and events or conditions that might exist in vivo. With the use of pure cultures of dissociated neurons there is a high level of control of experimental variables, which permits the investigator to accurately record or measure a variable of interest. Generally, culture models are subject to fewer assumptions than in vivo models. However, removing cells from their physiological environment removes them from a wide range of stimuli, which has the potential to greatly alter the function of the cell. The usefulness of cell culture studies is that they provide insights into possible physiological functions, insights that are not easily achieved from in vivo studies. Results from cell culture studies define how a cell can function under a set of experimental conditions, and not necessarily how the cell does function in the organism where conditions are different. In the following discussion of results, it should be self-evident that any dissimilarities between my results and previous observations in vivo might reflect the different experimental conditions.

Use of Iodinated Proteins

Previously reported procedures have been used in this thesis to iodinate LIF, NGF, and other proteins. Specific activities of the labeled proteins were calculated by cold trichloroacetic acid precipitation of an aliquot of each reaction mixture. The average specific activities were 2814 cpm/fmol ^{125}I-LIF (n=5) and 2109 cpm/fmol ^{125}I-NGF (n=15). The average specific activity of ^{125}I, based on the supplier's specifications (Amersham), was 5.1948×10^5 fmol/mCi,

which was equivalent to 3263 cpm/fmol ^{125}I, where the cpm was measured with the Wallac 1470 gamma counter. The ratio, moles ^{125}I/moles ^{125}I-protein, was calculated from the specific activities for ^{125}I and each of the labeled proteins and was found to average 0.9 and 0.7 for the monomeric forms of ^{125}I-LIF and ^{125}I-NGF, respectively. At this specific activity, ^{125}I-NGF has been shown by others to perform identically to non-iodinated NGF in receptor binding assays and bioassays (Herrup and Shooter, 1973; Sutter et al., 1979; DiStefano et al., 1992).

The volatility of ^{125}I is unlikely to have significantly affected our measurements of retrograde transport. The volatile species of iodine is diatomic elemental iodine (I_2) which forms from the oxidation of iodide (I^-) in acid solutions (Radiation Safety Manual, University of Alberta, 1996). In the iodination of proteins by lactoperoxidase, iodide is oxidized to monoatomic iodine which then bonds covalently to tyrosine residues and to a lesser extent to other amino acids. Iodine linked to proteins is relatively stable, but iodide or elemental iodine can dissociate from the protein over time. However, over the course of 24 hr (typical retrograde transport period) the release of iodine species from several iodinated proteins has been shown not to be appreciable (Bogdanove and Strash, 1975). The question of most relevance in the present experiments is whether the degradation products of ^{125}I-NGF might include elemental iodine which would then vaporize from the medium bathing cell bodies, thereby producing an error in quantiation of retrograde transport. In studies on several types of iodinated proteins and peptides, ^{125}I-tyrosine is a common degradation product, and our own findings on ^{125}I-LIF and ^{125}I-NGF degradation are consistent with this observation. The same studies also report the release of free iodide but not elemental iodine from the cells (Kuhlenschmidt et al., 1984; Sonne, 1985; Swope and Schonbrunn, 1987; Gandhi et al., 1993). While it is likely that some of the radioactivity released by neurons following degradation of ^{125}I-LIF and ^{125}I-NGF represents free iodide, it is unlikely that the iodide is oxidized to the volatile elemental iodine in culture medium (pH 7.4). Therefore, the total accumulation of radioactivity which we measured in each cell body/proximal axon compartment was likely a very close estimate of the true amount of ^{125}I-NGF retrograde transport.

LIF RETROGRADE SIGNALING AND TRANSPORT

Retrograde Signaling by LIF

LIF applied to distal axons induced changes in neurotransmitter enzymes in cell bodies/proximal axons, indicating that a retrograde signal was propagated to cell bodies/proximal axons. Conditioned media from cultured heart cells and dermal fibroblasts, known to contain cholinergic switching factors, also induced the cholinergic switch by a retrograde mechanism. The LIF retrograde signal varied as a function of the concentration of LIF applied to distal axons and as a function of the duration of the LIF treatment.

Despite the view that retrograde signaling is very prevalent and important for specifying neuronal phenotype and survival, there have in fact been few studies which have directly examined what specific molecules are capable of exerting a retrograde influence on neurons. Even for NGF, which represents the prototype of molecules capable of retrograde signaling, only a handful of studies have concretely demonstrated its retrograde effects. It should not be assumed that any ligand capable of evoking a response in neurons when administered to the entire neuron would be capable of evoking the response when administered only to axons. In this context the demonstration that a molecule with very different effects than NGF, can retrogradely signal is significant.

The findings suggest that LIF could be capable of exerting retrograde influences on sympathetic neurons in vivo. One possible condition where LIF might act in this way is in sweat gland innervation where axon terminals have been shown to undergo a developmental switch to a cholinergic phenotype. However, in the rat footpad where the cholinergic switch has been most commonly studied, the available evidence suggests that LIF is not the physiological switching factor (Rao and Landis, 1990; Rao et al., 1992). Instead, LIF might retrogradely signal following nerve injury (Rao et al., 1993), a condition in which mRNA levels in the nerve sheath increases markedly (Curtis et al., 1994; Banner and Patterson, 1994).

Retrograde Transport of LIF

LIF was retrogradely transported with similar characteristics to NGF transport. That is, the transport occurred by a receptor-specific mechanism, the accumulation of LIF in cell

bodies/proximal axons occurred with a similar time course to that previously reported for NGF, and LIF was partially intact in cell bodies/proximal axons under steady-state conditions. However, LIF was transported at lower levels than NGF. Also, LIF transport did not vary quantitatively across a 20-fold range in NGF concentration, suggesting that NGF did not regulate LIF transport.

While these studies were in progress, two groups reported that LIF is not retrogradely transported by sympathetic neurons in adult rats but that it is transported by sensory and motor neurons (Hendry et al., 1992; Curtis et al., 1994). One possible explanation for the contrasting observatons on sympathetic neurons between those studies and our study could be that younger neurons do transport LIF but adult neurons do not. Another possibility is that neurons in culture ("injured neurons") transport LIF but not uninjured neurons do not, since Curtis et al. (1994) found that peripheral nerve injury markedly increased LIF transport by sensory and motor neurons.

Our finding of LIF retrograde transport and of intact LIF in cell bodies/proximal axons establishes the possibility, in similarity to that proposed for NGF, that LIF-receptor transport could participate in retrograde signaling by LIF. It is intriguing that LIF and NGF were transported with similar time courses. One possible explanation is that the transport mechanisms for LIF and NGF were interdependent. For example, perhaps the LIF-receptor and NGF-receptor complexes were co-localized in the membrane and became encorporated into the same transport organelles. If this occurred, then it would be predicted that the level of LIF transport would change when the level of NGF transport also changed. However, LIF transport was found not to vary across a 20-fold NGF concentration range. A second possibility for the similar transport of LIF and NGF is that the time course of LIF or NGF transport was primarily a reflection of the kinetics of ligand binding, and that the similarity in time courses was a coincidence resulting from LIF and NGF receptors binding their iodinated ligands with similar kinetics. A third possibility, which seems most likely, is that the observation of similar time courses was not primarily a reflection of similar receptor properties, but rather was a reflection of generalized mechanisms in axons which determined the rate at which any transport-competent receptor would be internalized and transported. Examples of generalized mechanisms might be a limited number of sites in axons where internalization could occur, or secondly, a maturation of endosomes prior to loading onto the retrograde transport mechanism.

NGF RETROGRADE TRANSPORT

NGF Transport Velocity and Transport Lag

NGF was retrogradely transported at a velocity of 10-20 mm/hr. This speed is sufficient to permit NGF molecules to reach cell bodies within only 5 min of becoming associated with the axonal transport mechanism. However, in similarity to previous observations (Claude et al., 1982b), NGF was not detected in cell bodies until approximately 1 hr of distal axon incubation with NGF, indicating a lag in ^{125}I-NGF loading onto the transport mechanism.

A velocity of 10-20 mm/hr is much faster than previously reported. Studies on NGF transport by SCG neurons in vivo have estimated the transport velocity to be 2-3 mm/hr (Hendry et al., 1974a,b; Johnson et al., 1978), although in sensory neurons velocities of at 7-13 mm/hr have been reported (Stoeckel et al., 1975; Yip and Johnson, 1986). While it is possible that the discrepancy between my estimate and those in vivo might reflect real differences in transport velocity between younger, growing neurons and mature neurons in adult animals, I believe our experimental approach to be superior to that used in vivo. In vivo estimates were made by determining how long was required for ^{125}I-NGF to reach the SCG after injecting ^{125}I-NGF into the anterior chamber of the eye in adult rats. The ^{125}I-NGF is taken up by the mature axon terminals innervating the iris and transported over an estimated distance of 20 mm. The in vivo experiments do not control for the possibility of significant time being required for administered ^{125}I-NGF to become loaded onto the transport mechanism. For example, endogenous NGF bound to transport-competent receptors must first dissociate before the administered ^{125}I-NGF can associate with them. Our experiments control for events prior to ^{125}I-NGF becoming associated with the transport mechanism. A velocity of 10-20 mm/hr at 37°C is in fact predicted from a large number of studies which have measured retrograde organelle velocities in axonal preparations, mostly from sciatic nerve (see Chapter 4).

The delay of approximately 1 hr before NGF supplied to distal axons began to accumulate in cell bodies might reflect that NGF-receptor complexes reside in the axolemma for considerable periods prior to internalization. Another possible explanation is that newly-formed

endosomes containing NGF-receptor complexes pass through a maturation or sorting stage lasting close to 1 hr before being retrogradely delivered along microtubules. Our report is the first to suggest that the delay results from some variable other than the velocity of transport. In vivo, the interval that elapses before target-administered ^{125}I-NGF begins to retrogradely accumulate in cell bodies has been attributed only to the transport velocity.

NGF Transport Fidelity

During ^{125}I-NGF retrograde transport, only 3% of the ^{125}I-NGF delivered from distal axons accumulated in an intermediate axon compartment, either in an intact or degraded form. Since extensively degraded ^{125}I-NGF is released rapidly from cells, it is unlikely that any significant ^{125}I-NGF degradation occurred in axons, followed by retrograde transport of degradation products.

The result suggests that the fidelity of the transport mechanism for delivering intact NGF from axons to cell bodies is extremely high. This is an important finding, considering the perceived importance of the retrograde accumulation of intact NGF in cell bodies for retrograde signaling. An opposite finding, that a significant amount was degraded or released intact from axons, would have raised serious doubt about the retrograde signaling function of transported NGF. Inquiry into axonal degradation or release of neurotrophic factor from axons have been untested until this time because of the great difficulty in quantitatively addressing these questions in vivo.

NGF Degradation Rate

NGF was degraded at a rate of 40% of the cell body/proximal axon NGF pool per hour during steady-state incubation. By pulse-chase analysis, the average NGF half-life was 3 hr. The pulse-chase analysis also revealed that NGF was degraded at 2 more distinct rates, differing approximately 2-3-fold.

The observation of 3-hr half-life is in agreement with the previous observation of a 4.5-hr half-life in vivo (Korsching and Thoenen, 1985). The steady-state turnover rate which I observed

is similar to that previously reported for freshly dissociated sensory neurons (Sutter et al., 1979) and for PC12 cells cells (Layer and Shooter, 1983). A comparison of the turnover of NGF and other ligands in nonneuronal cells reveals that NGF is degraded slower than most other ligands, which is advantageous for maintaining a large NGF supply in cell bodies. No previous studies have reported heterogeneity in NGF degradation rates within a single cell type.

NGF Transport Rate

No more than 25% of the distal axon-associated NGF was delivered to cell bodies each hour. More commonly, transport rates averaged 10%/hr or less, which can be considered to be low. At least 85% of distal axon NGF appeared to be at the axon surface at steady-state, suggesting that the internalization rate was rate-limiting for NGF delivery to cell bodies. Slowly-dissociating receptors, whose binding strongly correlated with NGF retrograde transport, accounted for 20-60% of total NGF association to axons, suggesting that the low transport rate which was observed was not primarily due to a lack of transport-competent receptors on axons.

I consider the retrograde transport rate to be low, based partly on interpretations of higher internalization rates in PC12 cells (Layer and Shooter, 1983; Bernd and Greene, 1984) and partly on observations that NGF can be internalized within only several minutes of binding to surface receptors (Bernd and Greene, 1984; Kasaian and Neet, 1988; Buxser et al., 1990). Data presented by Bernd and Greene (1984) suggested an NGF internalization rate in PC12 cells of approximately 300% of the steady-state, surface-bound NGF per hour. However, since the cell harvest methods in that study might have eliminated low affinity binding, it is possible that 300%/hr represented the internalization rate of NGF bound to high affinity (slowly-dissociating) receptors. Steady-state NGF turnover in PC12 cells can be interpreted from data presented by Layer and Shooter (1983) to have been approximately 30% of the cell-associated NGF/hr, which suggests that the internalization rate would also have been approximately 30% of the surface-bound NGF/hr. This interpreted rate is still higher than the retrograde transport rates which I routinely observed. Electron microscopy studies on PC12 cells have provided conflicting reports about the internalization rate following initial application of labeled NGF (Hogue-Angeletti et al., 1982; Bernd and Greene, 1983). No reports are available from previous studies about how much axon terminal-associated NGF is transported per hour.

My observation that retrograde transport correlated with slow receptor (high affinity) binding is consistent with previous studies which reported that high affinity receptors are important for NGF transport in vivo (Dumas et al., 1979) and NGF internalization by PC12 cells (Bernd and Greene, 1984; Hosang and Shooter, 1987). The finding that transport rates were low even when slow receptors represented a significant fraction of all axonal receptors is consistent with the internalization rate being limited primarily by some variable other than the quantity of slow receptors. This conclusion is made because if all slow receptors could internalize NGF within several minutes of binding, as suggested from studies on PC12 cells, then retrograde transport rates should have been much higher than what was observed. A variable which might have limited retrograde transport is the availability of molecules necessary for internalization of ligand-receptor complexes. The finding of a similar time course of LIF and NGF retrograde transport (above) is consistent with this possibility. The possibility remains, however, that only a subset of slow receptors were in a state permissable for internalization and retrograde transport.

A low rate of retrograde transport would not be expected if target-derived trophic factor is in limiting quantity and neurons must efficiently transport it and concentrate it in cell bodies in order to survive. Alternatively, it is possible that the level of cell body NGF required to maintain a maximal cell body response might be sufficiently maintained by a low rate of retrograde transport. In any event, my finding highlights the need to directly test the hypothesis that NGF transport is important for retrograde signaling.

Distribution of Cell-Associated NGF

When ^{125}I-NGF was supplied continuously to distal axons, no more than 30% of neuron-associated ^{125}I-NGF was found in cell bodies, representing the retrograde accumulation, with the remainder being associated with distal axons. More commonly, less than 20% was present in cell bodies.

NGF quantities and concentrations per gram of tissue have been measured in vivo, with the original findings that NGF is much more concentrated in sympathetic ganglia than in sympathetic neuron targets such as the iris, heart, and submandibular gland (Korsching and Thoenen, 1983b, 1988; Nagata et al., 1987). While the in vivo observations might at first seem contradictory to our findings, the observations in vivo are in fact difficult to interpret. For

example, detectable NGF in target tissues could represent free, extracellular NGF, axon-bound NGF, or NGF mechanically released from secretory cells upon homogenization. It is not known whether the NGF in ganglia is evenly distributed among all neurons or whether the distribution is heterogenous. In addition, a recent study has reported that the original techniques of measuring tissue NGF failed to release NGF from high affinity receptors, and so NGF concentrations in some tissues are likely to be up to 10-fold higher than previously thought (Zettler et al., 1996). Thus, the in vivo experiments can not be directly compared to our experiments because significant assumptions must be made in interpreting the in vivo data and because the in vivo experiments did not directly examine the axon-association and cell body accumulation of NGF for one distinct set of neurons. Corroborating our findings, one recent study in vivo has indeed shown NGF to be extensively associated with axon terminals of at least some sympathetic neurons (Liu et al., 1996).

The extensive association of NGF with distal axon supports the likelihood that there is extensive signal generation occurring in axons. In turn, it raises the intriguing possibility that local signals generated by NGF binding to axonal receptors might serve to initiate a retrograde signaling mechanism that does not require the retrograde transport of NGF. Interestingly, other experiments from our lab have shown that phosphorylation of trkA in cell bodies/proximal axons occurs within 1 min of increasing the concentration of NGF bathing distal axons. This retrograde trkA phosphorylation is too rapid to result from vesicle-mediated transport of NGF-trkA complexes, considering that the NGF transport velocity was calculated as 10-20 mm/hr and a minimum transport distance for NGF to cell bodies/proximal axons is 1 mm. Therefore, it appears that non-vesicular types of retrograde signaling does occur in the neurons.

TrkA and p75 Function in NGF Transport

Blocking NGF binding to trkA with RTA antibody reduced NGF transport by 80% on average, indicating that trkA was very important for NGF transport and suggesting that p75 delivered a small amount of NGF to cell bodies. Blocking NGF binding to p75 with REX antibody reduced NGF transport by 60% on average, but blocking NGF binding to p75 with excess BDNF reduced NGF transport by only 25% on average. REX and excess BDNF blocked

NGF association with axons to similar levels, suggesting that their different effects on NGF transport did not arise because of differing degrees of blocking NGF binding to p75.

These results do not precisely delineate the quantitative contributions of trkA and p75 to NGF transport because of the possibility that RTA, REX, and/or BDNF influenced transport other than simply by blocking NGF binding to trkA and p75. However, the results do suggest that trkA is very important for retrograde transport that p75 does contribute to retrograde transport to some extent. It is possible that p75 might have even contributed to over half of the transport. Previous studies have not provided consistent results on a role for p75 in NGF transport. Curtis et al. (1995) reported that p75 binding was not significantly involved in NGF transport in adult sympathetic neurons in vivo. However, it has been reported that p75 is retrogradely transported in sympathetic neurons and other neurons (Taniuchi and Johnson, 1985: Johnson et al., 1987; Yan et al., 1988), and the MC192 antibody which increases NGF association with p75 (Chandler et al., 1984), augments NGF transport by sympathetic neurons (Taniuchi and Johnson, 1985; also unpublished observations).

The results that REX and BDNF exerted different effects on transport but not on ^{125}I-NGF association are intriguing because they suggest that p75 can regulate the transport of trkA-NGF complexes under some experimental conditions. Moreover, they suggest that the regulation occurs by a mechanism other than the control of the local NGF concentration, which has previously been presented as a model of p75-trkA interaction (Barker and Shooter, 1984). Rather, our results are more consistent with a newly proposed model of p75-trkA interaction which involves cooperative binding to p75. To further clarify how p75 and trkA participate in NGF transport (e.g. carrier versus regulatory functions), future studies should examine if bivalent and Fab forms of RTA and REX affect NGF transport differently. These experiments would help to determine the role of receptor crosslinking and activation in NGF transport.

BDNF Association to Axons

A 150-fold excess of unlabeled BDNF failed to compete ^{125}I-BDNF association with distal axons and even increased ^{125}I-BDNF association in older cultures.

The extensive BDNF/^{125}I-BDNF association with axons likely reflected positively cooperative binding of BDNF to p75 and also possibly binding to a very low affinity form of p75

or another receptor expressed at very high levels. Cooperative binding of BDNF and other neurotrophins to p75 has previously been reported although never to the degree which might have occurred in our experiments (Venkatakrishnan et al., 1990; Rodriguez-Tébar et al., 1990, 1992; Bothwell, 1995). Also, cooperative binding has rarely been factored into models of NGF receptor function. The coincidence of the odd effects of excess BDNF on ^{125}I-BDNF association and on ^{125}I-NGF transport raises the possibility that p75 allostery not only alters neurotrophin binding but might also regulate subsequent events (e.g. internalization) responsible for retrograde delivery of neurotrophins. The magnitude of the BDNF association also raises questions about what role cooperative binding might play in other cellular responses. Further studies should examine the concentration-range over which binding to p75 is cooperative and whether biological responses correlate with cooperative binding.

A MODEL OF NGF RETROGRADE TRANSPORT

The following model of NGF retrograde transport by sympathetic neurons takes into account the results obtained from these studies as well as speculations arising from these results and those of other investigators. The model is not meant to be solely a summary of observations but rather as an updated hypothesis of how NGF might be bound by axon terminals of sympathetic neurons and retrogradely transported. This model should serve as a basis for further experimentation.

Receptor Binding and Internalization:

- NGF binds to p75 and trkA on axons. Under steady-state conditions p75-NGF and trkA-NGF complexes are mostly at the axon surface.
- Most trkA-NGF complexes are linked to the cytoskeleton, making them relatively immobile in the membrane and producing a patchy distribution on the axons. Second messenger molecules and perhaps molecules which stabilize trkA-NGF dimers are also concentrated at the sites of trkA-cytoskeleton interaction. The interaction of trkA-NGF with the cytoskeleton and with the associated molecules produces slow dissociation characteristics.
- Most p75-NGF complexes are not linked to the cytoskeleton, making them relatively mobile in the membrane and producing a diffuse distribution along axons. However, some of the p75 does interact with the clustered trkA. The interaction is initiated through physical association

of the extracellular domains of the receptors but subsequently involves cross-receptor signaling through the intracellular domains. The p75 which associates with trkA is a high affinity dimer, a product of cooperative binding. The p75 dimers help to stabilize trkA dimers.

- NGF is internalized at the sites of cytoskeletal linkage, either through trkA dimers or through complexes of trkA dimers and p75 dimers. Most or all internalization requires trkA. Internalization does not occur at a high rate, indicating either that receptor dimers are short-lived and therefore unable to efficiently initiate receptor-mediated endocytosis or that other molecules required for efficient internalization are in limited supply.

Axonal Transport:

- From their axonal surface location NGF-receptor complexes are internalized as consituents of coated vesicles. Coated vesicles undergo maturation into transport organelles (e.g. smooth vesicles, multivesicular bodies). The maturation requires 30-60 minutes before transport vesicles are loaded onto the microtubule transport mechanism. NGF-receptor complexes are not re-routed back to the axonal surface by an energy-dependent mechanism.
- Vesicles carrying NGF-receptor complexes are trafficked to cell bodies at 10-20 mm/hr, the limit of axonal transport velocity. The transport vesicles do not acquire NGF degradative enzymes during transport. Thus, NGF-receptor complexes remain in a bound and intact state during transport. NGF-receptor complexes are not re-routed to the axonal surface during the shuttle of transport vesicles along microtubules.

Cell Body Accumulation:

- NGF-receptor complexes enter cell bodies but are not re-routed in significant numbers back into axons.
- NGF is degraded at a steady-state rate of 40% of the cell body NGF per hour. However, this rate represents an average of 2 different rates. Different rates might reflect the type of transport organelle which enters cell bodies which in turn might depend on the specific type of receptor-NGF complex being transported. NGF is degraded stepwise, resulting in the accumulation of intermediate degradation products. Partially degraded forms of NGF might

be attentuated in their ability to bind and activate organellar receptors. Highly degraded NGF (e.g. amino acid residues) exits cell bodies very rapidly.

RELATIONSHIP BETWEEN RETROGRADE TRANSPORT AND RETROGRADE SIGNALING

At least since the discovery of NGF retrograde transport over 20 years ago, retrograde signaling by neurotrophic factors has widely been theorized to require retrograde transport of the factor. This view has not been experimentally challenged, although some findings do raise question about its validity. For example, studies have reported that even though CNTF and basic FGF exert biological effects on some specific neurons, they are not retrogradely transported by those neurons (Ferguson et al., 1990; Smet et al., 1991). Conceptually, it is possible that retrograde signaling does not require that the trophic factor be transported. For example, activated second messenger molecules generated on axons might themselves be transported and signal in cell bodies. Another possibility is that the receptors might be activated by neurotrophin binding on axons but be able to retain their signaling function throughout their transport without having to remain neurotrophin-bound.

There have been several investigations in nonneuronal cells on whether ligand-receptor complexes serve their function while embedded in the plasmalemma or, alternatively, whether they must be endocytosed to exert their full range of effects. This question is somewhat analogous to whether retrograde transport of neurotrophin-receptor complexes is required for retrograde signaling. The primary effects of EGF and gonadotropin-releasing hormone have been reported to occur because of receptor activation at the cell surface without the need for internalization (Conn and Hazum, 1981; Chen et al., 1989; Wells et al., 1990). On the other hand, internalized EGF-receptor complexes are kinase-active (Cohen and Fava, 1985; Kay et al., 1986). Also, studies on other receptors suggest that internalized receptors do contribute to biological responses (Rothlein and Kim, 1983; Von Hoegen et al., 1989; Gilboa et al., 1995). Thus, whether a receptor is active within cells in addition to being active at the cell surface seems to depend on the receptor and the cell in question. In cylindrical cells it is possible that signals might be propagated from the cell surface to the nucleus without the need for intracellular shuttling of the ligand and receptor, but

in neurons in which axon terminals and cell bodies can be separated by tremendous distances this would seem less likely.

The results presented in this thesis do not directly address whether NGF-receptor complexes exert their effect only locally in axons or additionally whether their accumulation in cell bodies is responsible for retrograde signaling. However, our observations help us to speculate on the relative importance of NGF-receptors in local and retrograde signaling. The extensive, steady-state association of NGF with receptors on axons and the relatively low NGF transport rate are consistent with a prominent role of NGF-receptor complexes locally in axons. The known, local control of axon growth by NGF is an obvious consequence of receptor binding on axons. The high fidelity of NGF transport, its accumulation intact in cell bodies, and its relatively slow degradation are more consistent with retrograde signaling by transported NGF-receptor complexes. Future investigations will likely reveal that NGF-receptor transport is responsible for some, but not all retrograde signals.

REFERENCES

Barker PA, Shooter EM (1994). Disruption of NGF binding to the low affinity neurotrophin receptor $p75^{LNTR}$ reduces NGF binding to trkA on PC12 cells. Neuron 13:203-215.

Bernd P, Greene LA (1983) Electron microscopic radioautographic localization of iodinated nerve growth factor bound to and internalized by PC12 cells. J Neurosci 3:631-643.

Bernd P, Greene L (1984) Association of ^{125}I-nerve growth factor with PC12 pheochromocytoma cells. J Biol Chem 259:15509-15516.

Bogdanove EM, Stash AM (1975) Radioiodine escape is an unexpected source of radioimmunoassay error and chronic low level environmental contamination. Nature 257:426-427.

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 18:223-253.

Buxser S, Decker D, Ruppel P (1990) Relationship among types of nerve growth factor receptors on PC12 cells. J Biol Chem 265:12701-12710.

Claude P, Hawrot E, Dunis DA, Campenot RB (1982) Binding, internalization, and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J Neurosci 2:431-442.

Chandler CE, Parsons LM, Hosang M, Shooter EM (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 259:6882-6889.

Chen WS, Lazar CS, Lund KA, Welsh JB, Chang C-P, Walton GM, Der CJ, Wiley HS, Gill GN, Rosenfeld MG (1989) Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. Cell 59:33-43.

Cohen S, Fava RA (1985) Internalization of functional epidermal growth factor:receptor/kinase complexes in A-431 cells. J Biol Chem 260:12351-12358.

Conn PM, Hazum E (1981) Luteinizing hormone release and gonadotropin-releasing hormone (GnRH) receptor internalization independent actions of GnRH. Endocrin.109:2040-2047.

Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, Zhu Y, Lindsay RM, DiStefano PS (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: Correlation with increased LIF expression in distal nerve. Neuron 12:191-204.

Curtis R, Adryan KM, Stark JL, Park J, Compton DL, Weskamp G, Huber LJ, Chao MV, Jaenisch R, Lee K-F, Lindsay RM, DiStefano PS (1995) Differential role of the low affinity

neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14:1201-1211.

DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ (1992) The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8:983-993.

Dumas M, Schwab ME, Thoenen H (1979) Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. J Neurobiol 10:179-197.

Ferguson IA, Schweitzer JB, Johnson EM Jr (1987) Basic fibroblast growth factor: Receptor-mediated internalization, metabolism, and anterograde transport in retinal ganglion cells. J Neurosci 10:2176-2189.

Gandhi, CR, Harvey SAK, Olson MS (1993) Hepatic effects of endothelin: metabolism of {^{125}I]endothelin-1 by liver-derived cells. Arch Biochem Biophys 305:38-46.

Gilboa L, Ben-Levy R, Yarden Y, Henis YI (1995) Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interaction of neu receptors with coated pits. J Biol Chem 270:7061-7067.

Hendry IA, Stockel K, Thoenen H, Iversen LL (1974a) The retrograde axonal transport of nerve growth factor. Br Res 68:103-121.

Hendry IA, Stach R, Herrup K (1974b) Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. Br Res 82:117-128.

Hendry IA, Murphy M, Hilton DJ, Nicola NA, Bartlett PF (1992) Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. J Neurosci 12:3427-3434.

Herrup K, Shooter EM (1973) Properties of the β nerve growth factor receptor of avian dorsal root ganglia. Proc Natl Acad Sci USA 70:3884-3888.

Hogue-Angeletti R, Stieber A, Gonatas NK (1982) Endocytosis of nerve growth factor by PC12 cells studied by quantitative ultrastructural autoradiography. Br Res 241:145-156.

Hosang M, Shooter EM (1987) The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. EMBO J 6:1197-1202.

Johnson EM Jr, Andres RY, Bradshaw RA (1978) Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I]NGF. Br Res 150:319-331.

Johnson EM Jr, Taniuchi M, Clark HB, Springer JE, Koh S, Tayrien MW, and Loy R (1987) Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. J Neurosci 7:923-929.

Kasaian MT, Neet KE (1988) Internalization of nerve growth factor by PC12 cells. J Biol Chem 263:5083-5090.

Kay DG, Lai WH, Uchihashi M, Khan MN, Posner BI, Bergeron JJM (1986) Epidermal growth factor receptor kinase translocation and activation *in vivo*. J Biol Chem 261:8473-8480.

Korsching S, Thoenen H (1983) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Corrleation with density of sympathetic innervation. Proc Natl Acad Sci USA 80:3513-3516.

Korsching S, Thoenen H (1985) Treatment with 6-hydroxydopamine and colchicine decreases nerve growth factor levels in sympathetic ganglia and increases them in the corresponding target tissues. J Neurosci 5:1058-1061.

Korsching S, Thoenen H (1988) Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev Biol 126:40-46.

Kuhlenschmidt TB, Kuhlenschmidt MS, Roseman S, Lee YC (1984) Binding and endocytosis of glycoproteins by isolated chicken hepatocytes. Biochem 23:6437-6444.

Layer PG, Shooter EM (1983) Binding and degradation of nerve growth factor by PC12 phoechromocytoma cells. J Biol Chem 258:3012-3018.

Liu, D. T., Reid, M. T., Bridges, D. C. M., and Rush, R. A. (1996). Denervation, but not decentralization, reduces nerve growth factor content of the mesenteric artery. J. Neurochem. 66, 2295-2299.

Nagata Y, Ando M, Takahama K, Iwata M, Hori S, Kato K (1987) Retrograde transport of endogenous nerve growth factor in superior cervical ganglion of adult rats. J Neurochem 49:296-302.

Rao MS, Landis SC (1990) Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron 5:899-910.

Rao MS, Patterson PH, Landis SC (1992) Multiple cholinergic differentiation factors are present in footpad extracts. Develop. 116:731-744.

Rao MS, Sun Y, Escary JL, Perreau J, Tresser S, Patterson PH, Zigmond RE, Brulet P, Landis SC (1993) Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. Neuron 11:1175-1185.

Rodríguez-Tébar A, Dechant G, Gotz R, Barde YA (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Rodriguez-Tébar A, Dechant G, Gotz R, Barde YA (1992) Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J. 11:917-922

Rothlein R, Kim YB (1983) Two dinstinct mechanisms of cytotoxicity by porcine alveolar macrophages in antibody-dependent and immobized immune complex-dependent cellular cytotoxicity. J Immunol.131:1438-1443.

Smet PJ, Abrahamson IK, Ressom RE, Rush RA (1991) A ciliary neurotrophic factor from peripheral nerve and smooth muscle which is not retrogradely transported. Neurochem Res 16:613-620.

Sonne O (1985) Receptor-mediated degradation and internalization of insulin in the adenocarcinoma cell line HT-29 from human colon. Mol Cell Endocrin 39:39-48.

Stöckel K, Schwab M, Thoenen H (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: A biochemical and morphological study. Br Res 89:1-14.

Sutter A, Riopelle RJ, Harrris-Warrick RM, Shooter EM (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J Biol Chem 254:5972-5982.

Swope SL, Schonbrunn A (1987) Characterization of ligand binding and processing by bombesin receptors in an insulin-secreting cell line. Biochem J 247:731-738.

Taniuchi M, Johnson EM Jr (1985) Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directd against the rat nerve growth factor receptor. J Cell Biol 101:1100-1106.

Venkatakrishnan G, McKinnon CA, Ross AH, Wolf DE (1990) Lateral diffusion of nerve growth factor receptor: modulaton by ligand-binding and cell-associated factors. Cell Regul 1:605-614.

Von Heogen I, Falk W, Kojouharoff G, Krammer PH (1989) Internalization of interleukin 1 (IL 1) correlates with IL 1-induced IL-2 receptor expression and IL 2 secretion of EL4 thymoma cells. Eur J Immunol 19:329-334.

Wells A, Jwelsh JB, Lazar CS, Wiley HS, Gill GN, Rosenfeld M (1990) Ligand-induced transformation by a noninternalizing epidermal growth factor receptor. Science 247:962-964.

Yan Q, Snider WD, Pinzone JJ, Johnson EM Jr (1988) Retrograde transport of nerve growth factor (NGF) in motoneurons of developing rats: Assessment of potential neurotrophic effects. Neuron 1:335-343.

Yip HK, Johnson EM Jr (1986) Comparative dynamics of retrograde transport of nerve growth factor and horseradish peroxidase in rat lumbar dorsal root ganglia. J Neurocytol 15:789-798.

Zettler C, Zhou XF, Bridges D, Rush RA (1996) Increased concentrations of nerve growth factor detected in tissue extracts with an improved extraction procedure. J Neurosci Res (in press).